MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

I. INACTIVATION BY RDE AND BY VIRUSES OF THE INFLUENZA GROUP, OF THE SERUM INHIBITOR OF HAEMAGGLUTINATION.

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Francis (1947) recently described the high titre of inhibitor of heated B virus, characteristic of human sera. This inhibition did not depend on the presence of specific antibody. Francis' findings were readily confirmed in this laboratory and extended to other vertebrate sera. A further study of the phenomenon was undertaken in the hope that it would throw light on some aspects of haemagglutination. In the course of this work it was found that the "Francis inhibitor", as we have come to call it, was inactivated by preparations of the receptor-destroying enzyme (RDE) of *V. cholerae* as well as by the viruses of the mumps-influenza group. These findings we regarded as of importance, firstly in providing a substrate of the enzyme more convenient than the red cells with which all previous work had been done, and secondly in suggesting a relation between the Francis inhibitor and the red cell receptor.

The present paper describes these findings in detail and briefly discusses certain of their implications. In the following paper McCrea (1948) describes further properties of Francis inhibitor, properties which are compatible with its identification with serum mucoid. He finds that mucoid isolated from serum by standard methods is highly active as Francis inhibitor.

When it became probable that the inhibitor was a mucoid, the work of Burnet (1948) and McCrea (1948) on ovarian cyst mucoid in relation to viruses was initiated. This work, and the later phases of that reported in the present paper, proceeded in parallel, and their papers should be considered as forming one series with the present communication.

MATERIALS.

Fowl cells. Adult white Leghorn fowls were bled from the wing vein into citrate; the cells were separated and washed three times in normal saline.

Human cells. These were from group O adults. They were prepared by defibrination of the blood, and washing of the cells three times in normal saline.

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Serum. Human or animal blood was obtained from a vein and allowed to clot. Serum removed several hours later was heated at 56° C. for 30 minutes, then stored at 4° C.

Anti-RDE rabbit serum. Anti-RDE serum was obtained from rabbits ten days after the second of two subcutaneous injections of an emulsion of RDE of *V. cholerae*, and a lanoline and liquid paraffin base corresponding to Falba.

Tears. Tears were collected from the outer canthus of humans, using a blunt pipette.

Receptor-destroying enzyme (RDE). Unless specifically stated in the text, this was derived from an agar culture of *V. cholerae*, and heated at 55° C. for 30 minutes in the presence of excess calcium. The procedure has been described by Burnet and Stone (1947).

The RDE described in the text as an eluate from washed red cells, was prepared by adsorbing the above agar culture material at 4° C. on human cells, which were then washed in saline, and suspended at 37° C. in calcium borate buffer saline into which the RDE eluted.

Calcium borate buffer saline. This contained $CaCl_2$ 1·0 gm., NaCl 9·0 gm., H_3BO_4 1·203 gm., $Na_2B_4O_7.10H_2O$ 0·052 gm. per litre of distilled water (Burnet and Stone, 1947).

RDE-treated human cells (RD-H cells). These were prepared as described by Burnet and Anderson (1947).

Viruses. These were prepared as infected allantoic fluids harvested from 12-day chick embryos, and stored at 4° C.

NDV—Newcastle disease virus of the strain isolated in 1931 by Albiston and Gorrie (1942).

Mumps—An American strain kindly supplied by Dr. J. F. Enders.

Melbourne—Type A strain isolated in 1935.

WSE—An egg-adapted strain derived from the classical WS.

LEE—The classical B strain.

Swine—Shope's strain 15.

MIL B—A recent B strain isolated in Melbourne in 1945.

METHODS.

Absorption of Serum with Red Cells.

To a 10 p.c. dilution of serum, packed washed red cells of appropriate type were added to make a 10 p.c. suspension and stood at 4° C. for one hour. The cells and supernatant were separated by centrifugation.

Titration of Virus Haemagglutinin.

A modified Salk type pattern test was employed, using 1 p.c. red cells in normal saline in a total volume of 0·5 ml.

Titration of T Agglutinin.

Doubling dilutions of serum were prepared, and an equal volume of 1 p.c. RD-H cells added to each tube. The test was read after one and a half hours at 4° C. (Lind and McArthur, 1947).

Titration of Receptor-Destroying Activity of RDE.

The technique was that established by Burnet and Stone (1947) using fowl red cells and Melbourne virus as the test substances.

Titration of Nonspecific Inhibitor.

Falling doubling dilutions of serum were prepared in total volumes of 0·25 ml. with normal saline. Five agglutinating doses of MIL B virus were added, and then 1 p.c. fowl red cells in normal saline. The end-point was read at 20° C. by the deposited pattern of cells, the total volume in each tube being 0·75 ml.

Titration of Francis Inhibitor.

Falling doubling dilutions of serum were prepared in total volumes of 0·25 ml. with normal saline. Ten agglutinating doses of LEE virus, previously heated to 55° C. for 30 minutes, were then added, and the tubes stood at 4° C. for 60 minutes. Susceptible fowl cells at a dilution of 1 p.c. in 0·25 ml. normal saline were added, and the cell pattern read after 40 minutes at 4° C.

EXPERIMENTAL.

The Variation in the Titre of the Inhibition of Heated LEE Virus, according to the Cells Used.

The usual American practice is to use pooled cells from a number of fowls for haemagglutination work. We have always found it more convenient to use cells from individual birds, provided their suitability for the particular work in question had been established. On testing different fowl cells in titrations of Francis inhibitor, it was immediately evident that wide differences existed comparable with those observed by Anderson, Burnet and Stone (1946) in their work on nonspecific inhibition of active viruses. Table 1 shows that there is a complete parallel between the two types of reaction. The significance of this finding is discussed later. Human cells give consistently high levels when used in titrations of Francis inhibitor but owing to the convenience of rapid settling, fowl cells of the "high titre" type have been preferred for most experiments.

TABLE 1.

Inhibition by normal human serum of virus haemagglutination of fowl cells.

Cells from fowl number	Titre of inhibition of	
	Unheated LEE virus "nonspecific inhibition"	Heated LEE virus "Francis inhibition"
40	5	80
84	5	80
115	<5	30
211	8	40
118	100	6,000
185	100	3,000
207	40	1,600
219	80	4,800

The Inactivation of Francis Inhibitor, and of Nonspecific Inhibitor by RDE.

At the time when the work on Francis inhibitor was being commenced, other workers in this laboratory were investigating the factor in normal human serum which agglutinates red cells treated with RDE. This T agglutinin is present to varying titre in most adult human sera but absent from that of young infants (Lind and McArthur, 1947). It was thought of interest to determine whether the T agglutinin and the Francis inhibitor in serum might be identical. This could be decided by absorbing a serum with RDE-treated cells and seeing

whether the Francis inhibitor was removed. In the first exploratory experiment, cells treated with RDE were used without the normal washing after treatment with the enzyme. After absorption the titres of both the T agglutinin and the Francis inhibitor were found to have been reduced to low level.

Subsequent experiments done with fully washed cells showed only absorption of T agglutinin without change in the Francis inhibitor titre (Table 2). Further, three infantile sera containing no T agglutinin were found to contain Francis inhibitor equivalent in titre to that of normal adult sera. The discrepancy between the two sets of results was traced to the use of unwashed cells still containing appreciable amounts of RDE in the first exploratory experiment. Further work showed that brief incubation of serum with RDE regularly reduced the titre of Francis inhibitor, and to a lesser degree that of the nonspecific inhibitor of haemagglutination by active virus (MIL B).

TABLE 2.

Absorption of human serum by normal and by RDE-treated group O human cells at 4° C.

Serum treatment	Titre in treated serum of	
	T agglutinin	Francis inhibitor
Absorbed with normal cells	2,000	3,600
Absorbed with RDE-treated cells	80	3,600
Unabsorbed control	2,000	3,600

TABLE 3.

Inhibitor in serum: inactivation by RDE derived from V. cholerae.

A—at 37° C.

RDE		Titre of remaining Francis inhibitor		Titre of remaining nonspecific inhibitor	
Dilution	Titre	After 30 minutes	After 90 minutes	After 30 minutes	After 90 minutes
Undiluted	2,400	<50	<50	—	—
1:10	240	300	50	40	10
Saline control	0	4,000	4,000	60	60

B—at 4° C.

RDE		Titre of remaining Francis inhibitor		
Dilution	Titre	After 30 minutes	After 16 hours	After 72 hours
Undiluted	2,400	50	<20	<20
1:5	480	600	<50	<50
1:25	96	3,600	2,800	100
Saline control	0	4,000	4,500	4,500

A typical experiment establishing this point is as follows: 0·2 ml. of RDE, with a receptor-destroying activity of titre 2,400, was added to 1·6 ml. of calcium borate buffer saline, and to this 0·2 ml. of undiluted human serum. A control solution was prepared, using boiled RDE, and the same human serum. Both tubes were held at 37° C. in a water-bath for 30 minutes, and 0·5 ml. removed from each. The remainders were incubated for a further

60 minutes. The samples were set out in falling dilutions in saline, and titrated for their content of Francis inhibitor and of nonspecific inhibitor. As the Francis inhibitor is heat-stable even at 100° C., RDE may be removed from the system by heating to 65° C. for 20 minutes or to 100° C. for 5 minutes before this inhibitor is titrated. Similar results were obtained, whether or not RDE was thus destroyed prior to the titration of the Francis inhibitor.

We present the results of this experiment in Table 3A. The second half of the Table shows the results of a comparable experiment with Francis inhibitor and RDE carried out at 4° C. instead of at 37° C. At the lower temperature the rate of inactivation of the inhibitor was considerably reduced.

Identification of the Enzyme Inactivating Francis Inhibitor with RDE.

In the absence of a preparation of RDE in the form of a pure substance, one can support only by indirect evidence the assumption throughout this paper that the action on Francis inhibitor is due to the same enzyme as that destroying red cell receptors. The most cogent evidence is the correspondence in titres for the two activities of preparations which should contain quite different constituents other than enzyme. These are (1) agar culture filtrates which have been heated at 55° C. in the presence of calcium, to inactivate certain other enzymes (Burnet and Stone, 1947), and (2) eluates from washed red cells on which RDE had been adsorbed in the cold from preparations of type (1).

Table 4 shows the results of an experiment to compare the two activities of such preparations. It will be seen that the two preparations are of essentially equal activity by both types of titration.

TABLE 4.

Destruction of receptors, and inactivation of Francis inhibitor by RDE: comparison of titres.

Type of preparation of RDE	Titre of receptor-destroying activity	Titre of activity in inactivating Francis inhibitor
Agar culture filtrate of *V. cholerae* heated to 55° C.	1,750	700
Eluate from washed red cells	1,500	750

The titre of receptor-destroying activity was determined as described above. The power to inactivate Francis inhibitor was estimated as follows: the titre of Francis inhibitor in a sample of serum was determined (e.g. 1:1,500) and a standard solution prepared fifty times this concentration (e.g. 1:30). The preparation of RDE was set out in doubling dilutions in calcium borate buffer saline, in volumes of 0·25 ml., and to each tube 0·05 ml. of the standard solution of Francis inhibitor was added. The tubes were incubated at 37° C. for two hours, then held at 65° C. for 30 minutes to destroy the RDE. The presence of Francis inhibitor was determined in each tube by the addition at 4° C. of 10 agglutinating doses of heated LEE virus, and one hour later, of an equal volume of suitable red cells. In those tubes where the RDE had inactivated sufficient Francis inhibitor, heated LEE virus produced agglutination of red cells.

The Action of Influenza Virus Preparations in Inactivating Francis Inhibitor.

It has been a working hypothesis in this laboratory that the elution of influenza viruses from red cells is a function of an enzyme which is essentially equivalent to the RDE produced by the cholera vibrio. If this is so, it should be possible to show that virus preparations, like RDE, can inactivate the Francis inhibitor. In carrying out such experiments it is necessary

to remove the virus after it has been held in contact with the serum for an appropriate period (e.g. 3 hours at 37° C.) by heating the reaction mixture at or above 65° C. The mixture is then titrated for Francis inhibitor in parallel with a specimen of the same serum subjected to the same manipulations in the absence of virus. Well marked positive results were obtained in experiments of this type, and as will be seen from Table 5, there was in general an increasing activity as viruses successively further along the receptor gradient (Burnet, McCrea and Stone, 1946) were tested. As unconcentrated allantoic fluid virus was used in these experiments, it must remain a possibility that the inactivating action on the Francis inhibitor was due to an enzyme not actually incorporated in the virus particles. The different activity of different strains, however, makes it certain that the enzyme action is a function direct or indirect of the virus that gave rise to the active fluid. Further experiments are needed to clarify the relation of the enzymic action to the virus particles.

TABLE 5.

Francis inhibitor in rabbit serum: inactivation at 37° C. by virus and by V. cholerae *extract*

Serum dilution (1:10) treated for 2 hours with	Titre of Francis inhibitor in treated serum dilution
Saline	400
Mumps	400
NDV	240
Melbourne	200
WSE	240
LEE	30
Swine	15
MIL B	10
RDE 1:100	10

Titre of Francis inhibitor estimated against 10 agglutinating doses of LEE virus previously heated to 55° C. for 30 minutes.

Titre of RDE (1:100 dilution) was 24, determined according to the method described by Burnet and Stone (1947). The haemagglutinating titre of each of the viruses was adjusted to 200 with 1 p.c. human red cells before the experiment was commenced.

A Note on the Presence of Francis Inhibitor in Tears.

It was shown by Beveridge and Burnet (1945) that human tears had a high inhibitory effect on haemagglutination by the influenza A strain Melbourne. As this strain is almost insusceptible to inhibition by normal serum it was of interest to compare the activity of tears before and after treatment with RDE for 90 minutes at 37° C. Table 6 shows the results obtained in such an experiment using an active virus of strains Melbourne and LEE and the usual heated LEE virus used for determining Francis inhibitor.

TABLE 6.

Action of tears as inhibitor of haemagglutination.

Virus strain	Inhibitory titre of tears	
	Untreated	Treated RDE
Melbourne	1,500	120
LEE	70	<20
LEE (heated 55° C.)	2,000	60

The result confirms the earlier finding and shows that much of the activity is due to a component susceptible to inactivation by RDE. The very different pattern of inhibition from that produced either by serum or by the mucin preparations reported in the next paper, raises interesting possibilities of minor structural differences between the types of molecule capable of acting as influenza virus inhibitors.

DISCUSSION.

Three serum components are known to inhibit virus haemagglutination. The first is specific antibody of immune sera, presumably, like other antibodies, a gamma globulin. Its activity is specifically related to the virus type serving as antigenic stimulus. The second and third components are found in both immune and normal sera. The second is referred to in papers of this series as "nonspecific inhibitor" and is characterized by its inhibitory effect on *unheated* viruses. The inhibitory titre varies greatly against different virus strains and it is by no means established that only a single agent is concerned. The third is the Francis inhibitor, detected and titrated by its action on suitably heated influenza B viruses.

The chief significance of the work described in this paper is that it showed for the first time that the Francis inhibitor was rapidly inactivated both by the receptor-destroying enzyme (RDE) of *V. cholerae* and by influenza viruses. This allowed the development of a working hypothesis, on the basis of which the investigations reported in the subsequent papers were initiated. It had previously been postulated (Burnet, McCrea and Stone, 1946) that the influenza viruses carried an enzyme essentially similar to the soluble receptor-destroying enzyme of *V. cholerae*. The present finding suggested the hypothesis that the Francis inhibitor was also a substrate of these enzymes and therefore that it was of the same general chemical nature as the constituents of the receptor areas on erythrocytes or on cells susceptible to influenzal infection. A full discussion of the significance of the Francis inhibitor in relation to influenza viruses will be deferred to the final paper of this series (Anderson, Burnet, Fazekas, McCrea and Stone, 1948); but two other aspects of the present work will be considered here.

When cells from individual fowls were used as indicators of Francis inhibitor titre, those cells showing a high titre for Francis inhibitor show a high titre for non-specific inhibitor also, and there is in fact close correlation in the sensitivity of a virus to either inhibitor over a series of samples of fowl cells. Wide variations in the titre of nonspecific inhibition of an influenza B haemagglutinin over a range of fowl cells have been described by Anderson, Burnet and Stone (1946), who suggested that such variations are due to corresponding differences in affinity between virus and red cells. The correlation in sensitivity to Francis inhibitor and nonspecific inhibitor over a certain series of red cells is interpreted to indicate not that the two inhibiting agents are necessarily

similar, but merely that both show low affinity for the virus. Hence relatively small variations in the affinity between virus and red cells will be reflected in parallel variations in the titres of the two inhibitors.

The susceptibility of nonspecific inhibitor to inactivation by RDE suggested a possible application in the technique of Hirst type titrations of specific antibody. It should be possible by the action of RDE to eliminate interference by nonspecific inhibitor. No opportunity for a systematic investigation of this possibility has yet been found, but preliminary experiments showed that the specific antibody titre was also diminished by treatment with RDE in a small proportion of human immune sera. Any attempt to use the method would be premature without a comprehensive investigation of the complex problems involved.

SUMMARY.

The agent in normal serum which inhibits haemagglutination by heated LEE virus (Francis inhibitor) is inactivated by the receptor-destroying enzyme (RDE) of *V. cholerae*.

Living influenza virus preparations have a similar effect on the inhibitor, an effect which increases as viruses further down the receptor gradient are considered.

The factor in human tears which inhibits strain Melbourne is destroyed by the action of RDE.

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MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

II. ISOLATION AND CHARACTERIZATION OF THE SERUM MUCOID INHIBITOR OF HEATED INFLUENZA VIRUS

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Investigations in recent years have been made on agents in serum, other than specific antibody, which inhibit haemagglutination by viruses of the influenza group. In normal rabbit and ferret sera, for example, the inhibitory titre against certain influenza A strains may be of similar order to that of immune sera. With the great majority of influenza A strains, the inhibitor in normal serum may be inactivated by heating at 62° C., a procedure which leaves specific antibody titre relatively unchanged. The normal ferret serum inhibitor of BEL D is exceptional, however, in that it shows similar heat stability to specific antibody. From a comparison of the salting-out curves of specific antibody and nonspecific inhibitor, it has been concluded that the normal serum inhibitor, like antibody, is associated with the gamma-globulin fraction of serum (Burnet and McCrea, 1946; McCrea, 1946).

Francis (1947) has recently noted the exceptionally high inhibitory titre of serum against haemagglutination by heated influenza B virus. On further examination of this effect Anderson (1948) observed that the inhibitory titre against heated influenza B virus was rapidly lost when serum was incubated with the receptor-destroying enzyme (RDE) of *V. cholerae* (Burnet, McCrea and Stone, 1946; Stone, 1947).

Inactivation of the serum "Francis inhibitor" by RDE was obviously a finding of great interest in connection with current work in this laboratory on the possible enzymic action of influenza virus, as it suggested that a component in serum was similar to the specific substrate on the red cell. Chemical work on the direct action of RDE on intact red cells had been found unsatisfactory because, even with strict precautions and suitable controls, it was difficult to interpret results owing to a possibility of interference from slight haemolysis or diffusion of ions from within the cells (McCrea, unpublished data). Facilities were not available for the preparation of the purified receptor substance from red cells as described by de Burgh (1946).

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The present work, which was carried out in parallel with that of Anderson (1948), was undertaken to characterize the serum component described by Francis, and if possible to identify it as the substrate of RDE and virus enzyme. It has been shown that the Francis inhibitor is associated with serum mucoid, and that the characteristic inhibitory activity is lost on treatment with potassium periodate and proteolytic enzymes. Further, the mucoid inhibitor of heated virus can be differentiated from the nonspecific inhibitor of active virus. A preliminary note describing the identity of serum mucoid with Francis inhibitor has already been published (Burnet, McCrea and Anderson, 1947).[2]

MATERIALS AND METHODS.

Biological titrations. Francis inhibitor activity was estimated as described in the preceding paper by Anderson (1948). Doubling dilutions of sera or fractions were stood for 1 hour at 4° C. with an equal volume of heated LEE virus diluted to ten agglutinating doses. A further volume of 1 p.c. suspension of red cells from a selected Francis inhibitor-sensitive fowl was then added to each tube, and the agglutination pattern read after standing 60–90 minutes at 4° C. Where fractions were being titrated they were first diluted to the original serum volume.

Nonspecific inhibitor (normal serum inhibitor) of active virus was titrated as described previously (McCrea, 1946), using five agglutinating doses of the sensitive strain BEL D unless otherwise stated.

Enzymes. Crystalline trypsin, chymotrypsin, pepsin and ribonuclease had been received as a generous gift from Dr. Northrop; they were dissolved in saline immediately before use. Papain was prepared freshly for each experiment by extracting the powdered material with 100 times its weight of normal saline and filtering. Hyaluronidase was prepared from ram testes by Madinaveitia's (1940) method, and the activity measured by the mucin-clot prevention test (McClean, 1943). The "specific" form of choline esterase was prepared from human red cells as described by Mendel and Rudney (1943), the activity being estimated by direct titration with N/100 sodium hydroxide (Hall and Lucas, 1937).

Serum fractionation. Fractionation with ammonium sulphate and sodium sulphate was carried out as described previously (McCrea, 1946), the precipitates being separated on the "Ecco" high-speed centrifuge. The method of Pillimer and Hutchinson (1945) for estimating serum albumin-globulin ratios by precipitating with 42·5 p.c. methanol-water mixture in the presence of acetate buffer was adapted to precipitate various serum components by using varying methanol concentrations. The precipitates separated cleanly after ten minutes' centrifugation at 0° C. in a small angle centrifuge, and could then be washed with the appropriate methanol-water mixture containing a little acetate buffer, pH 6·5. After removing the supernatant fluid as completely as possible, the precipitates were dried overnight *in vacuo* at 0° C. before being reconstituted to the original serum volume in borate buffer pH 7·0. In the later stages of the work serum mucoproteins were isolated according to the methods of

[2] Whilst this paper was in press a publication by Hirst (J. exp. Med. 87, 301, 1948) became available. Hirst has confirmed all essential points concerning serum mucoid described in our preliminary note, namely heat-stability and inactivation by periodate, proteases, RDE, and virus. It is apparent, however, that Hirst confuses the heat-labile serum inhibitor of certain active influenza A strains with the inhibitor of heated influenza B, which, as stated in our preliminary communication, is stable at 100° C.

Rimington (1940) and Bray, Henry and Stacey (1946), with the slight modifications described in the text.

Nitrogen was determined by standard micro-Kjeldahl. pH determinations were made on the glass electrode.

EXPERIMENTAL.

Francis Inhibitor Titre of Various Sera.

The Francis inhibitor titre of sera from a range of species is shown in Table 1. Considerable inter-species variation exists, but it is clear that significant differences in activity occur from one species to another. Human serum is consistently higher than that of other species while horse, ox, and mouse serum are unusually low. With the exception of the rabbit, it will be seen that those species giving high serum titre also give red cells readily agglutinated by the influenza virus group: human and fowl cells are most suitable for agglutination work but horse, ox, and mouse cells are poor.

TABLE 1.

Francis inhibitor titre of sera from various species.

Serum	Titre against normal LEE "nonspecific inhibitor"	Titre against heated LEE "Francis inhibitor"
Human*	40	6,000
Rabbit	18	1,600
Sheep	5	1,280
Fowl	5	500
Horse	5	80
Ox	5	60
Mouse	5	35

* Serum of each type a pool from several individuals.

Fractionation of Francis Inhibitor from Serum.

From the point of view of preparing large amounts of active material from serum, it is unfortunate that those animals from which large volumes of blood can be obtained, such as the horse or ox, show very low serum titres. Some preliminary work with sheep serum was done, but it was found that, although the original activity is reasonably high, the inhibitory material appears more labile than that in rabbit and human serum. Consequently most of the experiments described were carried out with relatively small volumes of rabbit or human serum.

TABLE 2.

Fractionation of Francis inhibitor and nonspecific inhibitor from rabbit serum with ammonium sulphate.

Fraction	Francis titre	Inhibitor recovery p.c.	Nonspecific titre	Inhibitor recovery p.c.
Serum	1,400	100	70	100
33 p.c. saturation—precipitate	480	34	60	86
33 p.c. saturation—supernatant	560	40	<2	<1
50 p.c. saturation—precipitate	1,400	100	60	86
50 p.c. saturation—supernatant	10	1	<2	<1

Preliminary experiments with normal rabbit serum showed immediately that the Francis inhibitor activity is associated with the protein components. On precipitation with ammonium sulphate or sodium sulphate, the bulk of the Francis inhibitor activity was recoverable in the redissolved total globulin fraction (Table 2), but the activity did not appear to be entirely confined to the fractions from 33 p.c. saturation with ammonium sulphate (gamma-globulin) as was found with nonspecific inhibitor of unheated influenza virus (McCrea, 1946).

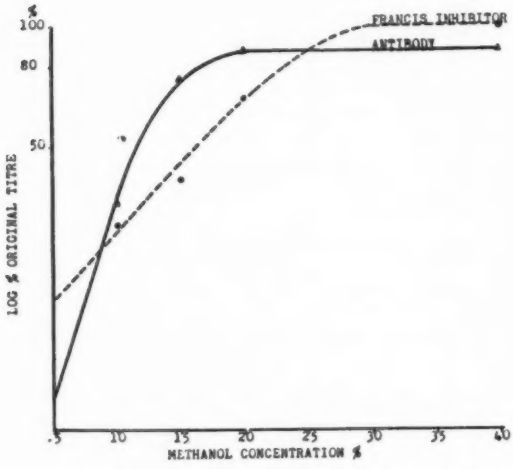

Fig. 1. Fractionation of Francis inhibitor and specific antibody (WSE) from rabbit serum with methanol-water mixtures.

Serial fractionation with the methanol-water mixtures as described previously showed that the Francis inhibitor activity could be recovered almost quantitatively at a final methanol concentration of 30 p.c. Again, however, slightly higher concentrations of precipitant were necessary for complete recovery when compared with the gamma-globulin nonspecific inhibitor, or with the gamma-globulin containing true antibody (Fig. 1). It will be seen from Fig. 1 that whereas maximum recovery of anti-WSE antibody is obtained at 20 p.c. methanol concentration, Francis inhibitor is not completely recovered until a concentration of 30 p.c. is reached. This difference may be considered too small for significance, but it was obtained consistently. At this stage it appeared that although Francis inhibitor was precipitated at similar salt and alcohol concentrations to the globulins, it could not be as clearly identified with a certain globulin fraction as could the nonspecific inhibitor.

Further observations emphasized the difference between Francis inhibitor and nonspecific inhibitor. Anderson (1948) had found that the Francis inhibitor titre of dilute serum was unaffected by heating, and in some circumstances actually increased. This has now been investigated in greater detail (Fig. 2).

Fig. 2 shows the titre of rabbit serum diluted in buffers of different pH value and heated at 100° C. Samples were removed at the intervals shown, cooled immediately to 0° C., readjusted to approximately pH 7 and titrated.

It will be seen from Fig. 2 that an approximately ten-fold increase in titre occurs when serum is heated at 100° C. for five minutes or longer at pH 7·4. When heated at pH 4·6, however, a sharp decrease in titre occurs during the first ten minutes, the titre then remaining relatively constant at 10–20 p.c. of that observed at zero time. The titre is reduced to approximately 50 p.c. on heating at pH 6·0 or pH 8·8. This behaviour is in contrast to that of the rabbit serum nonspecific inhibitor, which is inactivated after heating for several minutes at 62° C. and pH 7–8 (McCrea, 1946).

An increase in Francis inhibitor activity of similar order to that found on heating at pH 7–8 occurs when the total serum proteins are precipitated by alcohol-ether at —15° C., dried *in vacuo* and reconstituted to the original volume, or if the redissolved methanol-precipitated material is treated similarly (Table 3). For this reason it was found convenient

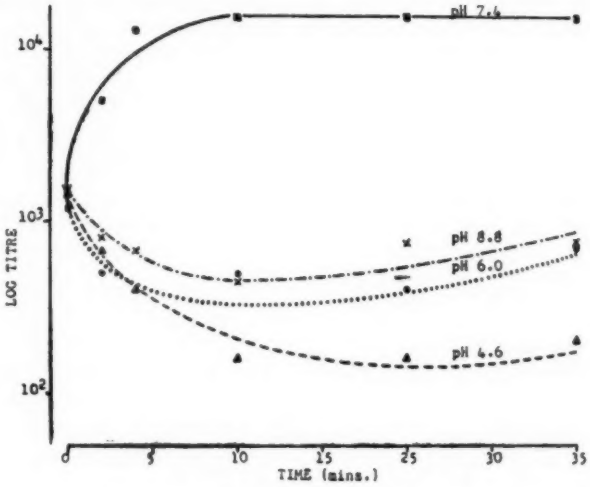

Fig. 2. Francis inhibitor titre of rabbit serum diluted 1 in 5 in buffers of various pH and heated at 100° C.

to prepare active semi-purified substrate for enzyme and other studies by first precipitating the active fraction with 20–30 p.c. methanol, redissolving in a small volume of saline or buffer, and then completing the lipid extraction with alcohol-ether. Vacuum-dried material prepared in this way maintained its Francis inhibitor titre for at least several months if stored in a desiccator. Further, as described in later sections, the inhibitor in this form was more susceptible to inactivation by enzymes and other reagents than was inhibitor in the form of untreated serum.

TABLE 3.

Effect of alcohol-ether precipitation at —15° C. on Francis inhibitor titre of rabbit serum.

Treatment	Francis inhibitor	Nonspecific inhibitor
Serum	1,600	120
Precipitated 25 p.c. methanol and redissolved	1,200	100
Methanol fraction precipitated alcohol-ether	4,800	60

Serum Mucoid and Francis Inhibitor.

The findings described above, showing that the Francis inhibitor is a heat-stable factor precipitated with the total globulin fraction, and in later sections showing that the inhibitor is rapidly inactivated both by proteases and by the carbohydrate reagent potassium periodate,

together with an observation at this stage made by Burnet (1948) that certain types of ovarian cyst fluid contain extremely high Francis inhibitor activity, suggested that the Francis inhibitor of serum might be associated with the serum mucoprotein.

Seromucoid was prepared from rabbit serum by the method of Rimington (1940), but with the slight modification that the heat-coagulated protein was removed by centrifugation: it was then washed several times with an equal volume of dilute acetate buffer at pH 4·7, since it was found that in each washing further Francis inhibitor could be recovered. Rimington did not wash the coagulum in his preparation and hence it is likely that his estimate of mucoid from serum is low. A typical preparation was made as follows. An equal volume of normal saline containing 1 in 1,000 merthiolate was added to 80 ml. of rabbit serum, and normal acetic acid added dropwise to pH 4·7. After heating to 80° C. for twenty minutes the precipitate was removed by centrifugation and resuspended overnight in 50 ml. acetate buffer pH 4·7. The protein was again centrifuged down, the washing with acetate buffer being repeated four times further. The washings and first supernatant fluid were then pooled and evaporated to small volume *in vacuo* at 55° C. After standing at 0° C. for 24 hours a further deposit was removed by centrifuging at 12,000 r.p.m. The product at this stage was an opalescent fluid which showed only a slight increase in turbidity on boiling or on the addition of 10 p.c. trichloracetic acid. The mucoid was precipitated by adding two volumes of alcohol-ether and standing at 0° C. for two hours. After drying *in vacuo* overnight a pure white powder remained in yield equivalent to 0·12 gm./100 ml. serum. This was redissolved in normal saline, dialysed in cellophane for several hours against distilled water, and a further slight deposit centrifuged down. A very faintly opalescent solution resulted, showing a very slight increase in turbidity with trichloracetic acid, a faint biuret reaction and a positive but typical slowly-developing Molisch reaction.

TABLE 4.

Francis inhibitor activity of rabbit seromucoid prepared by heating at pH 4·7.

Fraction	Francis inhibitor titre	Total N mg./100 ml.	Titre / N
Serum	2,400	1,084	2·2
Seromucoid	400	19	21·0
Coagulated Protein	240	944	0·2

Table 4 gives the activity of the seromucoid expressed as the ratio of Francis inhibitor titre to total N. It will be seen that the titre of the seromucoid is reduced to 15 p.c. as compared with the original serum titre. This is due to the inactivation of Francis inhibitor on heating in acid solution (Fig. 2), but in this respect it was felt necessary to adhere strictly to Rimington's method in order to identify Francis inhibitor with seromucoid. In spite of this low yield, however, it is apparent from the total N (and dry weight) of the fractions that the seromucoid is approximately ten times as active as the original serum, and approximately 100 times as active as the other protein components freed from seromucoid.

It is reasonable to conclude from these results that the Francis inhibitor of serum is associated with the mucoprotein.

Much higher recovery of active mucoid can be obtained by heating serum to 100° C. at pH 6·5. Table 5 records the titre of material prepared in this way.

50 ml. of human serum were adjusted to pH 6·5 with acetic acid, brought to 100° C. for one minute, and cooled immediately to 0° C. Acetic acid was then added to pH 4·8 and the coagulated protein centrifuged down at 2,500 r.p.m. The precipitate was washed with acetate

buffer at pH 4·7, the washings pooled with the supernatant fluid, and, after neutralization, concentrated to 10 ml. *in vacuo*. The pH of the concentrated fluid was again adjusted to 4·7, a further precipitate of protein then being centrifuged down at 15,000 r.p.m. As shown in Table 5, mucoid prepared in this way retains reasonably high titre, but is not completely free from contaminating protein. This protein, however, can be removed by treatment with chloroform as described in the following section.

TABLE 5.

Francis inhibitor activity of human seromucoid prepared by heating at pH 6·5.

Fraction and treatment	Francis inhibitor titre	Total N mg./100 ml.	Titre / N
Serum	3,200	1,506	2·1
Heated pH 6·5, centrifuged	1,800	128	14·1
Acidified pH 4·7 at 0° C.	1,200	106	11·3

Serum mucoid with high Francis inhibitor titre has also been prepared from rabbit serum by a slight modification of Sevag's method as described by Bray, Henry and Stacey (1946).

The dried serum fraction precipitated by 30 p.c. methanol was redissolved in borate buffer pH 7, and then shaken repeatedly at room temperature with the chloroform-butanol reagent to remove the globulins by denaturation. After shaking, the denatured protein was centrifuged down, and a sample of the supernatant removed for estimation of Francis inhibitor. It was found that the titre remained relatively constant for at least ten treatments (Table 6), during which the protein content steadily decreased; after 15 treatments the titre was 50 p.c. of that at the beginning of chloroform treatment, the total N being then reduced by 87 p.c.

TABLE 6.

Purification of Francis inhibitor (Serum mucoid) by chloroform treatment.

Fraction	Francis inhibitor titre		Nonspecific titre				Total N	Francis titre / N.	BEL titre / N.	Melb. titre / N.
		p.c.	BEL D	p.c.	Mel.	p.c.	mg./100 ml.			
Serum	3,200	100	640	100	80	100	1,222	2·62	0·53	0·66
30 p.c. methanol	1,600	50	560	88	40	50	405	3·98	1·38	0·99
Chloroform × 2	1,600	50	560	88	40	50	305	5·24	1·84	1·31
Chloroform × 5	2,000	63	220	34	25	31	196	10·20	1·12	1·28
Chloroform × 10	1,400	44	70	11	10	13	148	9·45	0·47	0·68
Chloroform × 15	800	25	25	4	2	3	54	14·80	0·47	0·37

Table 6 also illustrates the contrasting behaviour of Francis inhibitor and nonspecific inhibitor, the activity of the latter after two chloroform treatments decreasing in parallel with total N. The nonspecific titre did not fall at two chloroform treatments most probably because the protein precipitated up to this stage consists of the globulin other than gamma-globulin included in the 30 p.c. methanol fraction. With continued chloroform treatment the nonspecific titre fell steadily until, after fifteen treatments, it was only 3–4 p.c. of the original. Further, the ratio nonspecific titre : total N decreased through the fifteen treatments, but the

ratio Francis inhibitor : total N increased approximately six-fold as compared with the original serum. At this stage the Francis inhibitor appeared to be purified as far as is possible by this method.

If chloroform treatment was continued past this stage, the Francis inhibitor activity, which was already decreasing more rapidly than during the earlier part of the treatment, finally disappeared entirely. After twenty treatments, for example, the activity was only approximately 5 p.c. of the original, the total N then being 6 mg./100 ml. The significance of this effect will be discussed in later sections.

Rapid inactivation of Francis inhibitor occurred if the chloroform treatment was carried out in acetate buffer at pH 4·6 as originally described (Bray, Henry and Stacey, 1946), or if the methanol fraction was extracted with alcohol-ether at —15° C. before chloroform treatment. A similar loss occurred if the serum was first heated at acid pH to coagulate the albumin and globulin. Extraction of the dried methanol fraction with 90 p.c. phenol (Morgan and King, 1943) also inactivated the inhibitor, although this method can be used successfully to prepare mucoid with very high activity from ovarian cyst fluid (McCrea, 1948).

The possibility that Francis inhibitor is associated with the choline-esterase of the serum mucoprotein (cf. Bader, Schutz and Stacey, 1944) was eliminated by experiments which will not be described in detail. In summary, the very active "specific" choline-esterase prepared from red-cell stroma (Mendel and Rudney, 1943) and the nonspecific choline-esterase of mouse serum show only insignificant Francis inhibitor activity and, conversely, the very active Francis inhibitor present in ovarian cyst mucoid (Burnet, 1948) shows only very slight choline-esterase activity.

The Action of Proteolytic Enzymes on Francis Inhibitor.

The Francis inhibitor of rabbit serum is inactivated on incubation with crystalline trypsin, chymotrypsin and pepsin and by cyanide-activated papain, but not significantly affected by crystalline ribonuclease or testicular hyaluronidase.

Serum was diluted with an equal volume of buffer at suitable pH, a volume of enzyme solution added, and the mixture incubated at 37–40° C., in the presence of toluol. Samples were removed at the required intervals, heated at 100° C. for two minutes to inactivate the enzyme, and diluted out immediately in cold buffered saline, the Francis inhibitor titrations then being carried out in the refrigerator in the usual way. Control samples incubated without the enzyme were removed and titrated at the same time. Fig. 3 shows typical results.

It will be seen from the Figure that the Francis inhibitor activity of serum is reduced to approximately 7 p.c. of the original titre after incubation with crystalline trypsin for one hour at 40° C., and to approximately 15 p.c. of the original titre after incubation for the same time with crystalline chymotrypsin. The semi-purified material, precipitated by 30 p.c. methanol, extracted with alcohol-ether and reconstituted to give the original serum titre, is also inactivated by the enzymes, but at considerably greater rate. Cyanide-activated papain and crystalline pepsin (in acetate buffer pH 3) inactivate the inhibitor in serum and in the lipid-free material, but there is no significant reduction on incubation with crystalline ribonuclease or hyaluronidase (Table 7).

The Action of Potassium Periodate on Francis Inhibitor.

In the Annual Report for 1945 of the International Health Division of the Rockefeller Foundation it is stated that workers[3] in the Foundation laboratories had shown that potassium periodate at high dilution destroyed virus receptors on red-cells. This report of the destruction of influenza virus receptor-substance by periodate suggested a means of con-

[3] It is now known that this observation was made by G. K. Hirst.

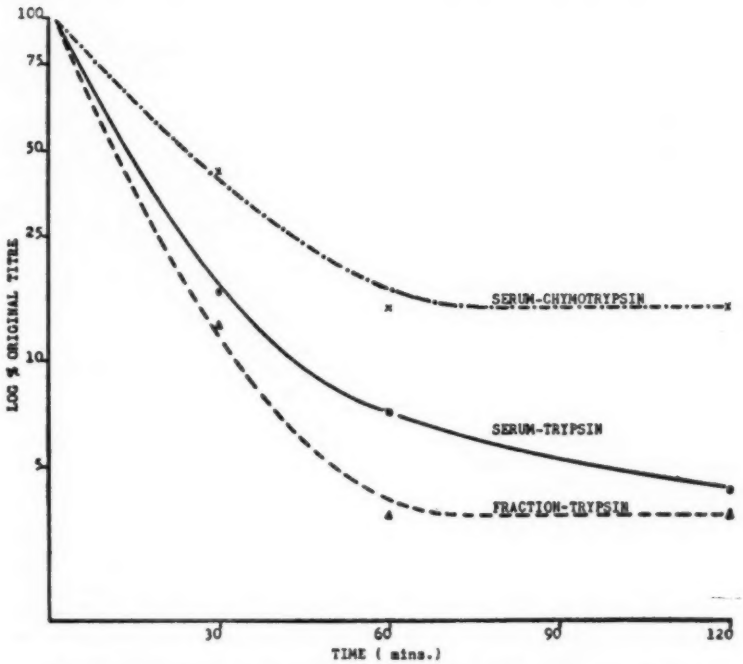

Fig. 3. Effect of proteolytic enzymes on Francis inhibitor titre of rabbit serum and methanol precipitated fraction. 40° C.
Enzyme concentration in serum = 1:200.
Enzyme concentration in fraction = 1:40,000.

TABLE 7.

Action of various enzymes on Francis inhibitor in serum and in 30 p.c. methanol precipitated serum fraction.

Enzyme *	pH	Serum		Fraction	
		Titre	p.c. control	Titre	p.c. control
Pepsin	3	160	33	<5	<1
Control	3	480		1,600	
Papain—KCN	7·5	140	25	<10	<1
Control	7·5	560		1,240	
Papain	7·5	480	100	750	48
Control	7·5	480		1,600	
Ribonuclease	7·5	280	87	1,600	100
Control	7·5	320		1,600	
Hyaluronidase	6·0	480	100	840	52
Control	6·0	480		1,600	

* All incubated 16 hours at 37° C.

firming our assumption that the Francis inhibitor of serum, was similar in structure to the cell receptor-substance.

It was found that the Francis inhibitor of serum was extremely sensitive to inactivation by very dilute periodate, particularly when the inhibitor was contained in the alcohol-precipitated and further purified material (Fig. 4).

Serum, the redissolved 30 p.c. methanol fraction, and fraction purified by chloroform treatment were added to an equal volume of M/15 phosphate buffer at pH 6, preliminary experiments having shown that at pH 6 the reaction proceeded approximately twice as rapidly as at pH 8. The periodate solution was then run in and rapidly mixed, samples being removed and titrated immediately in M/10 glycerol saline in the usual way. Two sets of pipettes were used to ensure that no traces of glycerol could reach the reaction mixture.

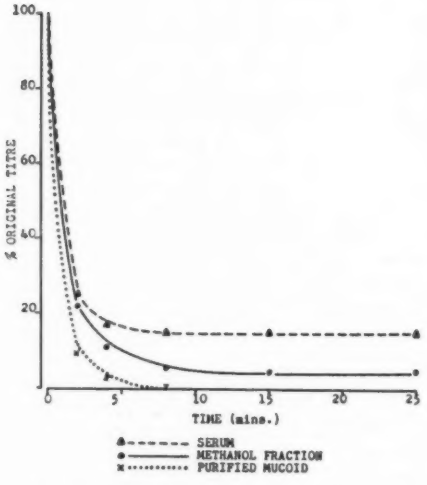

Fig. 4. Effect of M/1,500 potassium periodate on Francis inhibitor titre of (1) rabbit serum, (2) methanol-precipitated fraction, and (3) purified mucoid. 27° C.

The figure shows that even with M/1,500 final periodate concentration, and at room temperature, an extraordinarily rapid inactivation of Francis inhibitor takes place. The titres fall precipitately within two minutes, and thereafter remain relatively constant. A well-marked difference is shown in the degree of inactivation of inhibitor in the three forms: after four minutes, for example, the titres of serum fraction, and purified mucoid are reduced respectively to 17 p.c., 10 p.c. and 3 p.c. of those at zero-time.

These differences were found consistently in repeated experiments, the inactivation of purified mucoid always being extremely rapid even at concentrations of the order of M/4,000. In whole serum, however, a critical concentration must be reached before inactivation will occur: serum titre is unaffected if the experiment shown in Fig. 4 is repeated at M/2,000 periodate concentration.

Other oxidizing reagents, such as potassium perchlorate, potassium permanganate and hydrogen peroxide, are without effect on Francis inhibitor at molar concentration similar or even ten times greater than that found effective for potassium periodate. In a typical experiment, aliquots of serum were held at 18° C. with an equal volume of M/50 oxidizing reagents in phosphate buffer pH 6·0, titrations at the end of two hours showing that whereas the periodate-treated aliquot gave a titre of less than 10, aliquots treated with perchlorate, permanganate and peroxide were not reduced below the control treated with buffer only. If the concentration of permanganate was increased to M/20, however, significant loss of activity occurred in the more sensitive methanol-precipitated fraction (Table 8). Hydrogen peroxide also causes a slight decrease at this concentration, but it is clear that the activity of perchlorate, permanganate and peroxide is of an entirely different order to that of periodate. It may therefore be considered that the inactivation of Francis inhibitor by periodate is due to its specific action on carbohydrate (Jackson, 1944).

TABLE 8.

Action of periodate, perchlorate and other oxidizing reagents on Francis inhibitor in serum and in 30 p.c. methanol precipitated serum fraction.

Reagent	Final concentration	Serum		Fraction	
		Titre	p.c. control	Titre	p.c. control
Saline control*	—	400	100	2,800	100
Potassium periodate	M/1,000	100	25	10	<1
Potassium perchlorate	M/20	300	75	2,400	86
Potassium permanganate	M/20	350	88	350	12
Hydrogen peroxide	M/20	200	50	1,600	57

* All mixtures stood 1 hour at 21° C.

The Action of Potassium Periodate on Antibody and Nonspecific Inhibitor.

Several experiments were made to investigate the action of periodate on nonspecific inhibitor. It was found that this inhibitor is also inactivated by periodate, although to a significantly lesser extent than Francis inhibitor.

Serum was treated as before at pH 6 and room temperature, aliquots being removed and titrated in M/10 glycerol-saline. In the experiment illustrated in Fig. 5, serum from a rabbit immunized against WSE was treated with M/400 potassium periodate, specific antibody, nonspecific inhibitor (BEL D), and Francis inhibitor being titrated in parallel. It will be seen that both specific antibody and nonspecific inhibitor are inactivated to some degree by periodate at this concentration: the titre falls during the first five minutes, remaining constant thereafter at approximately 40–50 p.c. of the titre at zero time. As shown in earlier experiments, however, the Francis inhibitor titre falls precipitately within two minutes, then remaining constant at approximately 2 p.c. of the original titre. A similar differentiation between nonspecific inhibitor and Francis inhibitor can be shown with normal serum and M/1,000 periodate, but at this concentration specific antibody is not affected.

DISCUSSION.

The findings presented in this paper are compatible with the view that the Francis inhibitor of serum is a component of or is associated with the serum mucoprotein. The chief interest of the present work is that it demonstrated the existence in soluble form of a substrate for RDE and led to the identification of the general chemical nature of one such substrate in the serum mucoid fraction. The only other substrate recognized previously was that of the red cell "receptor."

It has been found subsequently that purified mucoid from ovarian cyst fluid and other sources shows considerably higher activity and stability than serum mucoid, and is thus more satisfactory for studies of the biological properties of RDE and virus strains (Burnet, 1948), as well as for purely chemical studies. For this reason the discussion at this stage will be limited

to a brief account of some of the chemical findings concerned with the relationship of serum mucoid to Francis inhibitor, and to the relationship of Francis inhibitor and the nonspecific inhibitor of active virus.

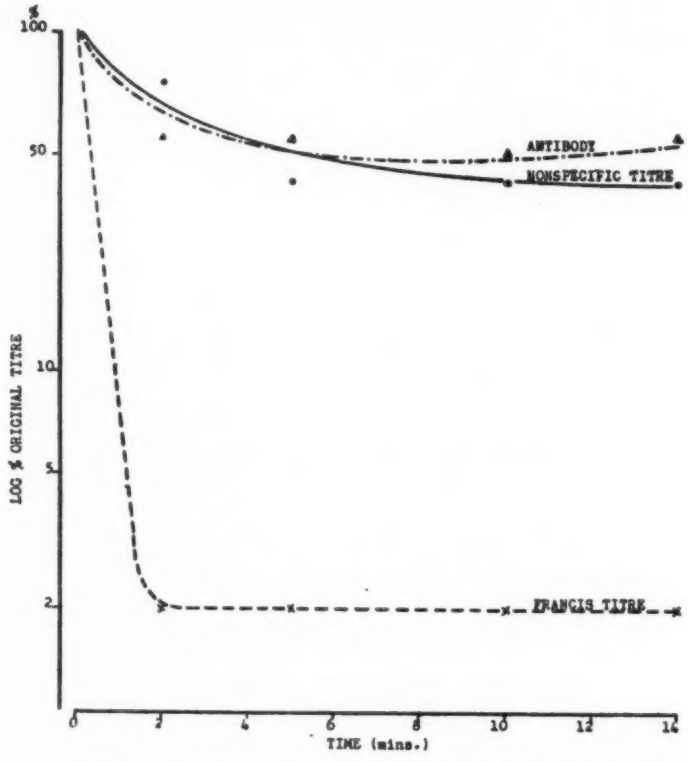

Fig. 5. Effect of M/400 potassium periodate on specific antibody (WSE), nonspecific inhibitor (BEL D), and Francis inhibitor in rabbit serum. 23° C.

The Francis inhibitor activity of rabbit and human sera, as described in the present paper, is clearly associated with the mucoprotein fraction. Serum "mucoid" purified by several methods shows, per unit of dry weight or nitrogen, considerably higher Francis inhibitor activity than that of the serum from which it is derived, and, further, the general properties of the inhibitor in regard to heat stability and inactivation by proteases and periodate are all in accordance with the known properties and structure of serum mucoids. Additional evidence may be seen in the findings, to be described in later papers, that highly purified

mucopolysaccharide with Francis inhibitor titre of the order of one in several million can be prepared from ovarian cyst fluid. The conclusion thus seems inescapable that the Francis inhibitor activity of body fluids is a function of certain mucoprotein or mucopolysaccharide components.

It is probable that only a small proportion of the molecules in the complex designated "serum mucoid" are responsible for the Francis inhibitor activity. In terms of dry weight purified serum mucoid is much less active than mucoids from other sources, a result which is of course partly due to the considerable loss of activity which occurs during purification. However, assuming that untreated serum with a titre of the order of 1,000 contains approximately 0·1 p.c. mucoid, the titre in terms of dry weight is still only of the order of 1 million as compared with 5–10 million for the most active ovarian cyst fluid.

No attempts have been made at this stage to determine the proportion of active Francis inhibitor material in the serum mucoid complex, or to identify the inhibitor with any one sub-fraction of the complex. Considerable confusion still exists on the nomenclature and homogeneity of various mucoprotein fractions, and also as to the state of their occurrence in native serum (e.g., Hewitt, 1939; Rimington and Van den Ende, 1940). As with all serum protein complexes, it is difficult to determine how far the various fractions obtained are the products of the chemical procedures by which they are isolated. On chemical and immunological grounds, however, it is likely that there are at least two main heat-stable mucoprotein fractions in horse serum, and possibly also other ill-defined peptide-carbohydrate components. Even carefully purified seromucoid, however, is not immunologically homogeneous (Rimington and Van den Ende, 1940). Hence we include our Francis inhibitor material under the general term "serum mucoid" without attempting further qualification at this stage.

An interesting aspect of serum mucoid, which it has not yet been possible to explore, is the possible correlation between variation in structure or composition and variation in inhibitory activity in sera from different species. Horse and ox serum mucoids, which are both associated with low Francis inhibitor activity, are the only serum mucoids studied in some detail. They are both stated to contain N acetylhexosamine, galactose and mannose, together with certain amino acids (Rimington, 1940; Rimington and Van den Ende, 1940). A comparative study of the carbohydrate and amino acid components of mucoids from different species does not appear to have been made. Precise chemical data on the composition of mucoids acting as Francis inhibitors will be of obvious interest in connection with current work in this laboratory on enzymic action of influenza virus.

In serum mucoid it is not yet clear whether the protein or the polysaccharide components of the complex are the more specifically concerned with Francis inhibitor activity. Whilst the experimental work was in progress, we considered

it likely that the inhibitory activity was a manifestation of the mucoprotein as such, i.e. that both protein and polysaccharide components of the molecule must be intact if the characteristic activity is to be retained. Evidence for this view was seen in the finding that serum Francis inhibitor is inactivated by proteases, and also inactivated by dilute potassium periodate, which under the conditions used in our experiments has been stated to be a specific carbohydrate oxidant. It has since been suggested that inactivation by periodate is not positive proof that carbohydrate is being attacked, as periodate is also capable of oxidizing certain amino acids such as threonine and serine. However, the optimum periodate concentrations used for reaction with amino acids are greatly in excess of those effective in our experiments. Further, hydrolysates or free amino acids are required for optimum periodate action on proteins (Martin and Synge, 1941; Shinn and Nicolet, 1941). It is thus difficult to interpret the inactivation of serum Francis inhibitor by periodate, although the very low concentration effective for serum and the similar inactivation of highly purified and active ovarian cyst mucoid, which contains only traces of amino acids, would perhaps suggest that the oxidation is more likely to involve the carbohydrate component.

There is little doubt that a relatively labile peptide or protein component is an essential part of the Francis inhibitor mucoid in serum. The rapid loss of activity on treatment with proteases can be due only to attack on peptide linkages. Further, inactivation by 90 p.c. phenol, by chloroform at acid pH, and by heating at acid pH are all compatible with the suggestion that a labile peptide component is an essential part of the molecule. The loss of activity which occurs when chloroform treatment is continued after the globulins have been removed may well be due to denaturation of the peptide components of the mucoprotein. All these properties are in contrast to those of the more stable ovarian cyst mucoid described in a later paper.

The question then arises as to the relationship between the nonspecific inhibitor and Francis inhibitor of any one serum. The nonspecific inhibitory effects of serum against haemagglutination by active (unheated) viruses were previously studied in more detail in ferret serum (Burnet and McCrea, 1946) and in rabbit serum (McCrea, 1946). It was shown that nonspecific inhibitory activity was associated with the gamma-globulin fraction. In rabbit serum the inhibitory activity was rapidly destroyed at 62° C., a property in distinct contrast to the relative heat stability of specific antibody. The experience of workers in this laboratory with inhibitory reactions of serum and mucous secretions indicates that apparent titres may vary widely according to the type of red-cell or strain of virus used, and also that a serum fraction containing all the inhibitory activity for a certain strain does not necessarily contain the same proportion of activity for a related strain. Further, even purified mucoid with high Francis inhibitor activity may still show moderate inhibitory activity against haemagglutination by some active viruses.

For these reasons, and for the complexity of the serum mucoprotein fractions as described earlier, it is advisable to retain the possibility that Francis inhibitor and nonspecific inhibitor activities may be functions of one protein-mucoprotein complex, the mucoprotein being capable of exerting its nonspecific inhibitory effect only in association with the heat-labile protein.

With these reservations, most of the data in the present paper would suggest that in serum the Francis inhibitor and nonspecific inhibitor are associated with independent components. The chief points of difference are: (1) Francis inhibitor activity withstands 100° C. for at least several minutes (and may actually increase if heated at appropriate pH), whilst nonspecific inhibitor is rapidly inactivated at 62° C. (2) Conversely, by using suitable concentrations of potassium periodate, Francis inhibitor may be rapidly inactivated but nonspecific inhibitor left relatively unchanged. (3) Slightly but consistently higher salt and alcohol concentrations are required completely to recover Francis inhibitor activity as compared with nonspecific inhibitor. (4) After treating serum by the Hardy-Gardiner process, Francis inhibitor activity increases approximately tenfold, which is not so for nonspecific inhibitor. (5) During isolation of Francis inhibitor by repeated treatment of serum with chloroform-butanol mixture, the ratio of inhibitory titre to total nitrogen increases with each successive treatment, whereas the ratio of nonspecific inhibitor titre to total nitrogen steadily decreases. Further, nonspecific titre becomes negligible at the stage where the bulk of the contaminating non-mucoid protein has been removed. (6) Anderson (unpublished) has found that it is possible to remove nonspecific inhibitor from serum by adsorption on red-cells without removing Francis inhibitor.

The five–tenfold increase in inhibitory activity observed when serum is heated to 100° C. in suitable conditions or extracted with alcohol-ether is a surprising and interesting finding, which it has not been possible to investigate fully. It may be that such treatment removes some suppressing agent, so allowing the true inhibitory titre to be revealed. Alternatively, partial denaturation of the peptide components of the mucoid macromolecule may expose further active groupings. Further experimental work would be required to support either of these hypotheses.

The inhibitory activity of mucoids and polysaccharides for haemagglutinins of other virus strains, and the biological significance of these inhibitors in the body fluids, will be discussed in later papers of this series.

SUMMARY.

The serum inhibitor of heated LEE influenza virus ("Francis inhibitor") has been identified as a component of the heat-stable mucoprotein fraction of rabbit and human sera.

On a dry weight basis, purified serum mucoid shows considerably higher activity than the serum from which it is derived. It is probable, however, that

only a small proportion of the molecules in the mucoid complex is concerned with Francis inhibitor activity.

The serum mucoid inhibitor is relatively labile in that it is inactivated by heat at acid pH, by chloroform treatment at acid pH, and by treatment with 90 p.c. phenol. On heating dilute serum at neutrality or extracting with alcohol-ether at —15° C. the apparent inhibitory titre may increase five–tenfold.

The inhibitory activity of serum is lost on incubation with trypsin, chymotrypsin, pepsin and cyanide-activated papain. Very rapid inactivation also occurs on treatment at room temperature with dilute potassium periodate. All these reactions proceed more rapidly with increasing purity of the mucoid fraction.

It is suggested that the characteristic inhibition can be shown only if the relatively labile peptide component of the mucoprotein complex is intact. The essential nature of the carbohydrate component is less clear.

The mucoid inhibitor of heated virus and the globulin inhibitor of active virus show many points of physical and chemical difference, but the possibility is still retained that both inhibitors may exist as a loose complex in native serum.

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MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

III. INHIBITION OF VIRUS HAEMAGGLUTINATION BY GLANDULAR MUCINS.

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Hirst (1942) showed that haemagglutination by influenza viruses was associated with adsorption of the virus to the cell surface which was subsequently followed by progressive reliberation of the virus. In the process the red cell lost its capacity to adsorb or be agglutinated by fresh virus. He recognized that the destruction of the adsorptive capacity of the cell was essentially similar to an enzymic action. It appeared that there was a definite cell component (receptor) to which influenza virus was readily adsorbed and which was subsequently destroyed by its action. A direct attempt to define this cell component has been made by Bovarnick and de Burgh (1947), who succeeded in obtaining from large amounts of human erythrocytes a lipid-polysaccharide which retained most of the original activity in inhibiting haemagglutination by PR8 virus.

Our own work has developed along entirely different lines and was initiated by the report of Francis (1947) that influenza B virus (LEE) on heating to 55° C. became extremely sensitive to inhibition by normal serum. As reported in a paper by Anderson (1948), it was found that the power of serum to inhibit heated LEE virus was destroyed by the action of the cholera vibrio enzyme (RDE). It has been shown by Stone (1947) that in all essentials the action of RDE on red cells is identical with that of influenza virus—a conclusion that has been greatly strengthened in other respects by subsequent work. Efforts were therefore made to isolate and characterize the serum inhibitor of heated LEE virus, which was finally identified as the serum mucoid (McCrea, 1948). For convenience we have referred to the substance or substances which act in this manner and are destroyed by RDE—as "Francis inhibitor" or F.I. While this work was in progress, Burnet and Stone (1947a) had under study another enzyme of *V. cholerae* which was an active depolymerase of various types of glandular mucin. A variety of circumstances, of which the most important was the finding of a statement in the 1945 report of the International Health

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

Division, Rockefeller Foundation,[2] that periodate destroyed the capacity of red cells to react with influenza virus, led to our testing mucins and related substances for F.I. activity. Several preparations, including highly purified blood group substances, showed high titre inhibitory activity which was rapidly destroyed by RDE. Further work has shown that a proportion of ovarian cysts (four of nine examined) give fluids with a high titre of F.I. and provide the most convenient source for its purification. The present paper is concerned with the inhibitory action of mucins and mucoids on active and heated influenza viruses, and with the destruction of this activity by RDE and by viruses. Preliminary notes on both these aspects have already appeared (Burnet, McCrea and Anderson, 1947; Burnet, 1947).

MATERIALS AND METHODS.

In the early stages of this work every effort was made to use true viscous mucins for all experiments in order to have available the test for cohesive quality (ACRA test) previously described (Burnet, 1948). As other workers have found, it is impossible to carry through the normal processes of protein purification with such mucins and recover the material in the original physical character. We found that a considerable degree of purification could be achieved by "dialysing" the mucin solution through filterpaper which is essentially impermeable to native glandular mucins. Among a considerable number of ovarian cysts that were studied one was found whose contents contained a high proportion of true mucin as defined by the production of a typical fibrous clot with acetic acid or with cetyl trimethyl ammonium bromide (CTAB) and a typical pattern at high dilution in acid alcohol. This material was diluted to a convenient level usually 1:20 and "dialysed" for four days in the cold against saline. The dialysed material usually had more than half the titre of the original both in regard to the ACRA test and the virus inhibiting power. Serological tests showed that human globulin present had been reduced to about an eighth of the original amount and added haemoglobin in other experiments was reduced to about the same level. No significant qualitative differences were observed between crude and "dialysed" preparations and a number of exploratory experiments were done with crude mucous secretions.

As the position became clearer more attention was paid to mucoids, particularly as through the kindness of Dr. W. T. J. Morgan pure samples of the mucoids concerned with A and O blood group specificity were available. Suitable material from pseudomucinous cysts could be prepared by diluting the contents with two volumes of M/5 NaCl adjusting if necessary to pH 6·5–7 and bringing to 100° C. for two minutes. This resulted in a heavy clot of coagulated protein and left the great bulk of the Francis inhibitor in the lightly opalescent supernatant fluid obtained by centrifugation. From the same cyst contents McCrea (unpublished) has obtained highly active material which appears to be essentially a pure mucoid. It has an inhibitory titre by our methods of about 1:4,000,000 in terms of the dried material. This has been used in some of the experiments included in this paper. It should be noted that solutions of the pure material show no significant differences in behaviour from the crude supernatant fluids and earlier experiments with these are included.

Virus Strains.

These are the standard influenza virus strains used in this laboratory. All have been frequently described. They are Influenza A (Melbourne, WS, BEL, Swine 15), Influenza B (LEE, MIL B), Newcastle disease (NDV) and Mumps (END).

[2] This observation was due to G. K. Hirst (personal communication).

RDE.

Preparations of receptor-destroying enzyme (RDE) were made by one of the two methods described by Burnet and Stone, i.e. either by heating an "agar filtrate" to 55° C. at pH 6 in the presence of excess Ca or by adsorption and elution of crude enzyme with human red cells. Most of that used in the current work was prepared by the first method, precipitated by saturation with ammonium sulphate, dialysed, and dried *in vacuo*. Stock solutions of 5 mg. per ml. had a titre of approximately 2,000 by the method of Burnet and Stone.

Virus Inhibitor Titrations.

Standard dilutions of allantoic fluid virus in saline were prepared to give five agglutinating doses with 1 p.c. fowl cells. The red cells were obtained from fowls known to provide suitable cells. As shown by Anderson, Burnet and Stone (1946) some fowls give cells which are very suitable for antibody titration since they show very low nonspecific inhibitory titres with normal sera. Others (a majority of birds) show relatively high inhibitory titres. These differences hold both for nonspecific inhibition of active influenza viruses and for the inhibition of heated influenza B virus haemagglutination (Anderson, 1948). Only birds known to provide cells of the second group were used as donors in this work. Human O cells will serve equally well and give titres almost identical with those obtained with suitable fowl cells, but the more rapid settling of the latter makes them preferable.

The solution of inhibitor is set out in doubling dilutions in saline over the desired range in 0·25 ml. amounts. An equal volume of standard virus dilution is added and after shaking left to stand for 30 minutes at room temperature. A third volume of 1 p.c. fowl cells is then added and the reading of cell deposit pattern made when settling of the cells is complete. As in all our work the end-point is taken as the "+" degree of partial agglutination.

When the action of virus or enzymes on the inhibitor was being tested, the mixtures were held at 37° C. for the required period and then heated to 65° C. for 30 minutes to destroy the active agent. With intact mucins there was sometimes a distinct fall in titre as a result of this treatment but not sufficient to cause any difficulty in the interpretation of results. Mucoids were unaffected and with these the heating at 65° C. could be conveniently replaced by heating to 100° C. for one minute.

Details of the ACRA test have been described (Burnet, 1948). Briefly serial dilutions of the mucin are coloured with Congo red and a drop of each let fall into acid alcohol. Characteristic patterns result and the endpoint is taken as the highest dilution giving a partially coherent pattern.

EXPERIMENTAL.

Experiments with Crude or Semipurified Mucin.

Most of the work to be reported was done with one of three types of preparation (a) "dialysed" mucin from the ovarian cyst referred to above, (b) pure blood group O substance (Morgan), (c) semipurified mucoid from pseudomucinous ovarian cysts.

A general picture of the inhibitory qualities of mucin can be obtained from the experiment with "dialysed" cyst mucin shown in Table 1. Four sets of mucin diluted 1:100 in buffered saline were prepared. These were treated as follows: (1) untreated and kept in the refrigerator, (2) heated at 65° C. for 30 minutes, (3) treated with 1:40 RDE for 1 hour at 37° C. and heated to 65° C. for 30 minutes, (4) as (3) but not heated at 65° C., titrated in the cold with M/120 "Calgon" (sodium hexametaphosphate) added to overcome the action of residual RDE. Each sample was then titrated against normal and heated (52° C. or 55° C.) virus of the strains shown, to determine its inhibitory activity.

The figures given in the Table are from mucin samples 1 and 3 which were not heated to 65° C. The heated specimens gave virtually identical figures with one exception. Heated MIL B virus gave an inhibitory titre of 2,400 with mucin treated with RDE. The probable explanation of the discrepancy is that the unheated sample of mucin with RDE was left in the refrigerator overnight before being titrated so that there was a further opportunity for slow action by the enzyme. The inhibitory titre with heated LEE B virus was not influenced.

TABLE 1.

Inhibitory effect of human cyst mucin on different influenza viruses.

Virus	Heated	Inhibitory titre with mucin	
		Untreated	After RDE action
Melbourne A	—	1,200	<200
"	55° 30′	2,400	<200
BEL A	—	150	
"	55° 30′	400	
Swine 15	—	600	<200
"	52° 30′	6,400	<200
LEE B	—	400	<200
"	55° 30′	200,000	6,400
MIL B	—	300	
"	55° 30′	100,000	<200

It will be seen from this Table that the mucin has a moderate inhibitory titre of a few hundreds against unheated viruses and a very much higher titre against the heated B viruses. The inhibitory titre against all types of virus was abolished or greatly reduced by treatment of the mucin with RDE.

TABLE 2.

Inhibitory effect of various mucinous substances of human origin on different influenza virus strains.

Strain		Cyst mucin	Pseudomucin G. crude	Purified mucoid G.	Respiratory mucin (crude)	B. G. S. O.
Melbourne A	N	1,200	100	<10	400	<1,000
	H	2,400	40	<10	400	<1,000
WSE A	N	..	40,000	400	300	<1,000
	H	..	60,000	30,000	1,500	100,000
Swine 15	N	600	300		40	<1,000
	H	6,400	2,000		1,800	..
LEE B	N	400	360	<100	30	<1,000
	H	200,000	32,000	20,000	1,500	600,000

In Table 2 are collected the inhibitory titres of a representative series of mucins and mucoids against four selected viruses. The main features are the relatively high inhibitory titres of intact mucins against unheated viruses while the mucoids were only effective against heated influenza B or WSE viruses.

The Action of RDE.

It can be stated explicitly that all the inhibitory effects of the various mucin and mucoid solutions on haemagglutination by heated or unheated viruses of the influenza group, can be reduced to 1 p.c. or less by the action of RDE.

Destruction of inhibitor to the lowest dilution tested was observed with all the materials included in Table 2 and in addition with several samples each of human gastric and uterine mucous and of guinea-pig intestinal mucin, with crude egg white and with partially purified ovomucin.

The action of RDE was similar irrespective of whether it was in the form of crude filtrate treated as described previously by heating in the presence of excess Ca, or preparations partly purified by elution from red cells or by ammonium sulphate precipitation and dialysis. The action of RDE was prevented completely when an active anti-RDE immune serum was added to the system.

Experiments to illustrate these points can be taken from work carried out with purified blood group substances kindly supplied by Dr. W. T. J. Morgan. Stock solutions in distilled water (2 mg. per ml.) were prepared of: (1) Blood group A substance from human ovarian cysts; (2) Group A substance from hog stomach mucin; (3) O substance from human ovarian cysts. These solutions were tested without addition and after addition (to 0·5 ml.) of 1 drop (0·025 ml.) of a stock RDE preparation of titre 1,400. The control and treated solutions were first held at 37° C. for 1 hour to allow the action of RDE and then heated to 65° C. for 30 minutes. Titrations for Francis inhibitor were made in the usual manner with the results shown in Table 3.

TABLE 3.

Action of pure blood group substances as Francis inhibitor.

Material	F.I. titre	
	Untreated	Treated with RDE
A human	15,000	<1,000
A pig	<1,000	<1,000
O human	200,000	<1,000

Further experiments with the more active group O substance may be cited to show the effect of varying concentrations of RDE, and of anti-RDE serum. The set-up was similar except that RDE diluted 1:10 and 1:100 was used as well as undiluted material and an additional two tubes received two drops (0·05 ml.) each of anti-RDE serum R228–3. In these tubes the enzyme and serum dilutions were held at 37° C. for 30 minutes to allow interaction before the O substance was added. In all other respects the procedure was the same as in the experiment just described.

It will be seen that the activity of RDE falls off fairly rapidly on dilution and that the addition of immune serum completely inhibits the action of the enzyme. The higher titre with the serum mixtures is a protective effect of the serum against the action of heat on the inhibitor.

Other experiments showed that group A substance of human origin, despite complete removal of its F.I. activity by RDE, had its full activity as an inhibitor of agglutination of group A human cells by an anti-A immune serum.

TABLE 4.

Action of O substance as Francis inhibitor.

O substance	RDE	Serum 228	F.I. titre
1:1,000	nil	nil	200,000
1:1,000	1:20	nil	<2,000
1:1,000	1:200	nil	80,000
1:1,000	1:2,000	nil	250,000
1:1,000	1:20	1:10	600,000
1:1,000	1:200	1:10	700,000

Destruction of Francis Inhibitor by Virus.

Anderson (1948) and McCrea (1948) have shown that the Francis inhibitor in serum is destroyed by active influenza virus. This holds also for inhibitors in the form of mucin or mucoid solutions. Two experiments may be cited. The first experiment was made with a "dialysed" preparation of ovarian cyst mucin at a dilution equivalent to 1:40 of the original material. An equal volume of allantoic fluid virus harvested within seven days and adjusted to a standard fowl cell agglutinating titre of 240 or 24 was added to 1 ml. lots of diluted mucin. Dilutions were in borate buffered saline, pH 7·1. A drop or two of chloroform was added as a bacteriostatic agent to each tube. The corked tubes were placed in a water bath at 37° C. and samples removed at the required intervals. These were diluted 1:10 in saline and heated to 65° C. for 30 minutes to destroy the virus. They were then titrated as usual for Francis inhibitor.

TABLE 5.

Destruction of Francis inhibitor by influenza viruses.

Virus	Haemagglutinin titre	Titre of Francis inhibitor after			
		2½ hours	5 hours	22 hours	46 hours
Melbourne A	240	250	160	60	
	24			40	20
LEE B	240	<40	<20	<20	
	24			<20	
Swine	240	64	20	<20	
	24			160	200
Saline control	0		600	600	100

The chief features of this experiment are (1) the evidence that the process by which the Francis inhibitor activity of mucin is destroyed progresses with time; (2) the different activity of different strains with the same haemagglutinin activity, LEE being much more active than Swine or Melbourne; (3) the virtual absence of further effect beyond 22 hours.

The second experiment with purified blood group O substance was carried out similarly, the final dilution of the pure substance in the reaction mixtures being 1:1,500 while the viruses used were at a final concentration equivalent in each case to 160 agglutinating doses. The mixtures were held overnight (17 hours) at 37° C.

TABLE 6.

Action of influenza viruses on purified blood group substance.

Virus	Francis inhibitor titre after action of virus	
Melbourne	30	(45,000)
Swine	3·0	(4,500)
LEE B	2·5	(3,700)
Control	240	(360,000)

More comprehensive experiments on the destruction of inhibitor by influenza viruses have recently been carried out. These will be the subject of a separate paper.

Action of Representative Crude Preparations of Mucin.

Three samples of human mucinous secretions that have been tested have shown activity as F.I. to about 500 times the dilution which was taken as "+" by the ACRA test. In each instance it was shown that RDE destroyed the activity to the lowest dilution tested. Only one of these preparations, RIC, was an inhibitor of group A blood cell agglutination. This activity was uninfluenced by the enzyme.

TABLE 7.

Human mucous secretions as Francis inhibitor.

Mucous secretion	ACRA titre	F.I. titre	
		Control	RDE treated
QVI pseudomucinous cyst of ovary	320	250,000	<200
GRI fasting gastric contents	30	15,000	<150
RIC cervical (uterine) mucous	20	20,000	<200

It is not yet possible to provide conclusive evidence that the same molecules are concerned in inhibiting heated virus and in producing the characterizing physical properties of mucin. All the circumstantial evidence points in this direction but proof will have to wait on chemical studies with some suitable type of mucous secretion.

The Action of Potassium Periodate on Mucin.

It has been reported from the Rockefeller Foundation Laboratories (Report, 1945) that dilute potassium periodate solutions destroyed virus receptors on the red cell. This has been confirmed and it was further shown by McCrea (1948) that the Francis inhibitor in serum was also destroyed by similar low concentrations. As would be expected mucin and mucin derivatives are also inactivated as Francis inhibitors by this reagent. As described elsewhere (Paper IV) periodate is capable of changing the physical character of native mucin at very high dilutions. Like RDE small concentrations of periodate have no effect on the blood group specificity when this is present. The following experiment is typical.

Potassium periodate was added to give a final concentration of M/1,000 to solutions of ovarian cyst mucin (approximately 1:200 of dense original material), cervical mucin (also approximately 1:200 of the original), blood group O substance and blood group A substance both prepared by Dr. W. T. J. Morgan and used at a dilution of 1:2,000. All the reagents were of human origin. The mixtures were held at 37° C. for one hour and then titrated for relevant characteristics. The same materials in the absence of periodate were treated similarly.

TABLE 8.

Action of KIO_4 on human glandular mucin and derivatives.

Reagent	ACRA test		Francis inhibitor		Agglutinin inhibitor	
	Control	KIO_4 treated	Control	KIO_4 treated	Control	KIO_4 treated
Cyst mucin	1	<1	500	<2	...	...
Uterine mucin	8	<1	>1,250	100	80	80
B.G.S. O.	...	...	240	<2		
B.G.S. A.	...	...	5	<2	500	500

DISCUSSION.

This paper is primarily concerned with showing that a variety of glandular mucins and mucoids are active inhibitors of haemagglutination by influenza viruses, particularly of heated influenza B strains. In all instances this inhibitory power can be completely destroyed by the same enzyme preparations from *V. cholerae* which are known to remove receptors from red cells and to prevent adsorption of virus and infection in susceptible cells of the allantoic cavity (Stone, 1948) or the mouse lung (Fazekas de St.Groth, 1948). In each instance tested the inhibitory power is also destroyed in enzymic fashion by allantoic fluids containing influenza virus B. A full discussion of the implications of these findings is given in the final paper of this series.

The present work on crude mucous secretions is necessarily of a preliminary character, since no suitable methods are available for the purification of glandular mucins with retention of their essential physical character. In any

given instance it is open to question whether the inhibitory effect was actually due to the mucin in the preparation or to some associated substance. The uniformity of the findings with human mucous secretions taken along with the absence of inhibitor in similar secretions of swine or bovine origin, and such other considerations as the effect of periodate, can leave no real doubt that in certain mammalian species at least a number of glandular mucins and mucoids have a common feature responsible for the inhibition and susceptible to enzymic destruction by the RDE of cholera vibrio or by active influenza B virus.

An interesting feature which suggests the desirability of a much wider survey of mucous secretions than we have yet been able to make, is the limitation of active preparations to the three species—man, guinea-pig and fowl—which provide cells readily agglutinated by influenza viruses. Mucin of the hyaluronic acid type, as represented by human synovial fluid, is quite inactive.

SUMMARY.

Crude mucous secretions of human origin and semipurified preparations of mucin from these are active inhibitors of haemagglutination by influenza viruses. The action is particularly marked against heated influenza B viruses.

Purified mucoids from certain ovarian cysts including pure blood group substances are very potent inhibitors of heated influenza B virus, but are virtually ineffective against active viruses.

The inhibitory effect is destroyed (a) by dilute periodate solutions; (b) by the action of the cholera enzyme, RDE; (c) by the enzymic action of influenza viruses, the strain LEE B being the most active of those tested.

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MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

IV. INHIBITION BY PURIFIED MUCOID OF INFECTION AND HAEMAGGLUTINATION WITH THE VIRUS STRAIN WSE

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In a previous paper (Burnet, 1948) it has been noted that the influenza strain WSE behaved in some respects like an influenza B strain in that haemagglutination by heated virus was inhibited to high titre by a specimen of purified mucoid. In addition, haemagglutination by unheated virus is also susceptible to high titre inhibition by certain preparations.

As this is the only established strain of influenza virus in our possession which can be inhibited to high titre in the active state, it was of interest to see whether mucoids could also inhibit infection by this strain. An incidental observation in the course of work on the action of periodate on mucoids showed that minimal action of this reagent very greatly increased the inhibitory power against WSE haemagglutination. Simultaneously the power to inhibit infection was also greatly increased.

MATERIALS AND METHODS.

The strain WSE, a derivative by chorioallantoic passage of the standard WS strain of influenza A, has been frequently described (Burnet, 1937). It has been used throughout in the form of allantoic fluid. Most of the work has been done with material from a large ovarian cyst (GER) but exactly similar results were obtained with the contents of another cyst (WH_2). The purified mucoid described was isolated from a pool of material from these two cysts. Many experiments were done with the supernatant fluid obtained by diluting the cyst fluid 1:3 in M/5 NaCl adjusting to about pH 6–6·5 and heating to 100° C. for two minutes. A heavy coagulum formed which separated well from the highly opalescent supernatant fluid. This "semipurified mucoid" had a high (10,000) inhibitory titre and in all relevant respects behaved identically with a solution of equivalent titre of the purified mucoid subsequently used.

Methods of titrating inhibitory power against heated LEE virus (Francis inhibitor) or against other strains of virus have been described in the preceding papers. Mouse and chick embryos were inoculated by standard methods.

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

EXPERIMENTAL.

The Action of Heat on WSE.

Fresh allantoic fluid virus, pH 7·8, of haemagglutinin titre (1 p.c. fowl cells) 840 was heated undiluted and without added buffer to the temperatures shown for thirty minutes. The haemagglutinin titre was then determined and appropriate standard dilutions prepared. These were used to titrate a preparation of "semipurified" mucoid which had been diluted to give a titre with heated LEE virus of 1,000.

TABLE 1.

Action of heat on strain WSE.

Temperature	Haemagglutinin titre	Inhibitory titre of S.P. mucoid
45° C.	850	10
47·5° C.	640	50
50° C.	500	500
52·5° C.	360	450

Table 1 shows that the capacity to be inhibited begins to appear at 47·5° C. and is fully established at 50° C. Preparations routinely used were heated to 52° C.

Action of Small Concentrations of Periodate on the Inhibitory Action of Mucoid on WSE.

It has been previously shown (Burnet, 1948) that periodate in low concentration destroys the Francis inhibitor of serum or of various mucins and mucoids. In the course of experiments to confirm this action when WSE was used as the virus to be inhibited, an interesting anomaly was encountered which appears to be sufficiently important to be reported. The phenomenon was quantitatively similar with semipurified solutions from WH_3 and GER and with the purified mucoid.

The following experiment with the pure material is typical. Mixtures were prepared in borate buffered saline pH 7·0 with a final concentration of F.I. of 0·025 p.c. equivalent to 1,000 inhibitory units and the molarity of KIO_4 shown. After thirty minutes at 37° C. a drop of glycerol solution (equivalent to about M/20 glycerol in the final mixture) was added to prevent any further action of KIO_4. The mixtures were then titrated in the usual fashion using both heated LEE and unheated WSE viruses.

TABLE 2.

Action of KIO_4 on Francis inhibitor and on inhibitor for WSE.

Concentration of KIO_4	Inhibitory titres against:	
	Active WSE	Heated LEE
M/2,500	120	10
5,000	500	65
10,000	640	400
20,000	400	500
Nil	60	640

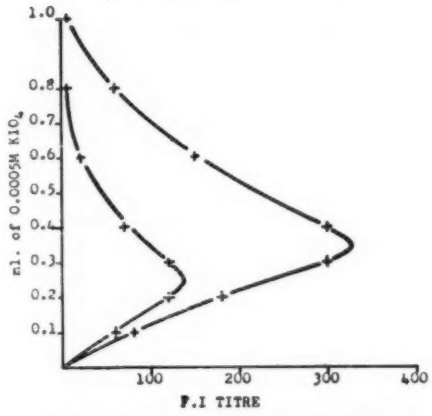

Fig. 1. Inhibitory titre against WSE of mucoid treated with graded amounts of KIO_4. For details see text.

In Fig. 1 is shown the result of a more detailed experiment in which two different concentrations equivalent to F.I. 2,500 and 1,000 of semipurified mucoid WH_3 were tested against a series of KIO_4 solutions in buffered saline. In the graph the ordinates show the volume of M/2,000 KIO_4 added to a total volume of 1·25 ml. and the abscissae show the inhibitory titre against unheated WSE. It should be noted that both scales are linear. There is a sharp inflection on both curves, on that which is equivalent to a 0·025 p.c. solution the inflection is at approximately M/10,000 with the stronger 0·062 p.c. mucoid at M/7,000.

The shape of the curve strongly suggests that under the conditions obtaining in these experiments a reaction involving one molecule of KIO_4 per mucoid unit and giving rise to an effective inhibitor is followed by successive reaction which destroys the inhibitor.

Fig. 2 which is representative of six essentially concordant experiments with semipurified material shows the relation of inhibitory action against active and heated WSE and active and heated LEE. The material used was an intermediate step in the purification of GER cyst mucin used at a final concentration of about 2,000 units (approximately 0·05 p.c.). The features common to all experiments were (1) almost complete destruction of all inhibitory effect when a concentration of M/2,000 was reached, (2) a maximum inhibitory effect against active WSE between M/5,000 and M/10,000, (3) a minor and almost equal inhibitory effect against active and heated LEE with mucoid treated with relatively large concentrations of periodate.

In most experiments with cruder material this third effect was more striking than in the two experiments cited in Table 2 and Fig. 1. In three experiments the first inhibitory effect against active LEE appeared with M/1,000 periodate and reached equivalence with the heated LEE titre at M/200, the titres lying between 80 and 160.

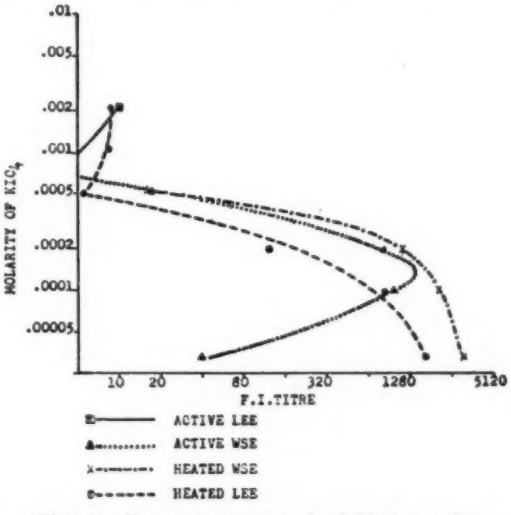

Fig. 2. To show changes in inhibitory titres against active and heated LEE and WSE viruses associated with treatment with periodate at different levels.

It is evident therefore that the action of periodate can modify the specific adsorptive activity of a soluble mucoid. Minimal action, possibly a single molecular change in the effective polysaccharide unit, results in a very sharp increase in inhibitory power for active WSE. Further action destroys inhibitory power for both types of WSE and for heated LEE in parallel. Then a third action becomes evident in the appearance of a low grade inhibitory power for both active and heated LEE.

Inhibition of Infection by Mucoid.

A preliminary experiment using semipurified GER mucoid was made in which serial dilutions of fresh allantoic fluid virus WSE were made in saline or in mucoid solution of F.I. titre approximately 10,000. After standing at room temperature for around thirty minutes, inoculations were made in mice intranasally and by the allantoic route in chick embryos. The inhibitory effect of the mucoid solution on the virus used was slight (1:40); on the same virus heated to 52° C. it gave a titre of 24,000.

TABLE 3.

Inhibition of infection by mucoid.

Mouse lung lesions					
Virus dilution		Control		Mucoid	
10^{-5}		4^7 3 3 3	(3·3)	2 2 1 0	(1·3)
10^{-3}		2 2 2 2	(2·0)	0 0 0 0	(0·0)
Allantoic infection					
Virus dilution		Control		Mucoid	
10^{-6}	2d	+ + + +	(4/4)	— — — —	(0/4)
	3d			+ — — —	(1/4)
10^{-5}	2d	+ + + +	(4/4)	+ ± — —	(2/4)
	3d			+ + + +	(4/4)

Mouse lung lesions according to usual convention:
4^7 = death on 7th day; 0 = no lesion.
+ = positive haemagglutination in allantoic fluid on day shown.

This experiment indicated a significant effect corresponding roughly to a hundredfold decrease in infectivity. Further experiments showed that this effect occurred regularly with high concentrations of the order of 24,000 F.I. (i.e. about 0·5 p.c. solution) and that it was greatly increased when mucoid was treated with appropriate amounts of KIO_4 to produce a high level inhibitor of haemagglutination.

In the following experiment a comparison was made of virus WSE in normal diluent (10 p.c. normal horse serum saline) in untreated mucoid solution and in the same solution treated (a) with periodate at 0° C. and showing high inhibition of unheated WSE and, (b) with twice the concentration of periodate at 27° C. resulting in complete destruction of inhibitory power. Excess of glycerol was added to all mixtures to neutralize any excess of periodate. Virus dilutions were held at 0° C. for approximately thirty minutes before inoculation into the allantoic cavity.

TABLE 4.

Inhibitory activity of mucoid solutions used.

Treatment	Inhibitory titre N.WSE	H.WSE
A. 2 ml. mucoid + 6·5 ml. PO_4 saline	200	16,000
B. 2 ml. mucoid + 0·5 ml. M/100 KIO_4 5 minutes 0° C.	8,000	14,000
C. 1 ml. mucoid + 1 ml. PO_4 saline + 0·5 ml. M/100 KIO_4 40 minutes at 37° C.	10	10

In the experiment "C" was mixed with half the concentration of virus used in the other series and twice the volume inoculated to give equivalent amounts of virus and treated mucoid per egg. The result of the four series of titrations is as shown.

TABLE 5.

Inhibition of infection in the allantoic cavity by treated and untreated mucoid.

Diluent		Virus dilutions inoculated in volume of 0·05 ml.							
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	Log ID_{50}
N.H.S.S. Control	1d	3/6	3/6	5/6					7·5
	2	4/6	5/5	5/6					7·8
	3	5/6		6/6					8
	4	5/6							8
Mucoid "A" Untreated	1d	0/6	0/6	0/6	0/6				<5
	2	0/6	3/6	5/6	6/6				6·9
	3	0/6	4/5	5/6					7·4
	4	1/6	4/5	6/6					7·5
Mucoid treated KIO_4 "B"	1d				...	...	...	...	
	2				0/6	2/6	2/6	4/6	2·7
	3				0/6	5/5	6/6	5/5	4·5
	4				1/5				4·6
Mucoid treated Excess KIO_4 "C"	1d	0/4	3/7	5/6					6·8
	2	2/4	7/7	6/6					8·0
	3	2/4							8·0

Figures show number of eggs giving fluids with positive haemagglutination over number with embryos alive on day shown.

It will be seen that the action of the mucoid in preventing infection runs very closely parallel to its effectiveness in inhibiting haemagglutination by the living virus. The experimental results are shown graphically in Fig. 3.

DISCUSSION.

An adequate interpretation of these results must obviously wait on the chemical elucidation of the structures in mucoids which are responsible for the power to inhibit haemagglutination by influenza viruses. The most interesting feature is the very narrow range of periodate concentrations which produces a maximal effect in increasing the inhibitory power of cyst mucoid for active WSE virus. As suggested above, this may mean that the point of maximal activation corresponds to the action of one equivalent of periodate per mucoid molecule and that further action by periodate results in changes which rapidly diminish the adsorptive power of the mucoid for any virus active or heated.

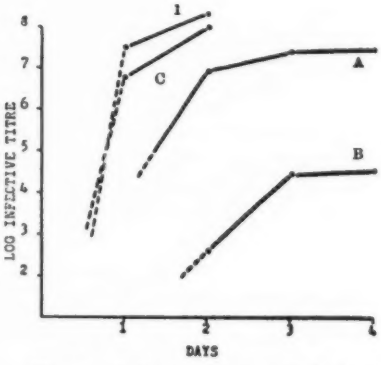

Fig. 3. Influence of mucoid treated with periodate in preventing infection of chick embryos. The 50 p.c. infective titre as determined by positive haemagglutination is shown for each day of incubation.

(1) Control in horse serum saline; (A) Dilutions in untreated mucoid; (B) Dilutions in mucoid treated to optimal level with KIO_4; (C) Dilutions in mucoid treated with excess of KIO_4.

The power of mucoid, treated to the optimal point with periodate, to prevent infection in chick embryos is evidence that, although haemagglutination is much more readily inhibited, the power to infect is affected by the same agents at higher concentration. This is in line with the finding of Burnet and McCrea (1946), that some normal ferret sera with high nonspecific inhibitory effect on haemagglutination showed power to prevent infection by the corresponding strain.

These experiments were carried out in parallel with more extensive studies of the action of periodate on the cell receptors which react with influenza viruses (Fazekas de St.Groth—to be published). Fazekas de St.Groth found that at certain levels of periodate action red-cell receptors retain their power to adsorb influenza viruses, but have become insusceptible to the secondary enzymatic action which results in elution of the virus from normal cells. How far this insusceptibility to enzyme action is associated with the effects observed in the present work must also remain unanswered until more chemical information is available.

SUMMARY.

Haemagglutination by the influenza virus A strain WSE is inhibited to low titre by purified cyst mucoid.

Treatment of the mucoid with small amounts of periodate greatly increases its inhibitory action, both against haemagglutination and against infection in the allantoic cavity.

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MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

V. THE DESTRUCTION OF "FRANCIS INHIBITOR" ACTIVITY IN A PURIFIED MUCOID BY VIRUS ACTION

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Previous papers (Anderson, 1948; McCrea, 1948; Burnet, 1948a, 1948b) have been concerned with the activity of various mucins and mucoids as inhibitors of haemagglutination by heated influenza B virus and with the destruction of this activity by the enzyme RDE from *V. cholerae* and by the enzymic action of active influenza viruses. The cell receptors concerned in haemagglutination and the initiation of infection are almost certainly areas of mucopolysaccharide composition, which actively adsorb the virus and are subsequently modified by its enzymic action. It should be possible therefore to obtain useful information in regard to the cell surface-virus interaction by studying the simpler but essentially analogous interaction between virus and a soluble mucoid. In a forthcoming paper McCrea (1948b) has described the preparation from ovarian cyst fluid and the properties of a mucoid which appears to be a virtually pure substance with an inhibitory titre against heated LEE virus of 3 to 4 million.

By following the changes in Francis inhibitor titre under the action of RDE or viruses it is possible to obtain a quantitative expression of enzymic activity and so to compare the activity in this respect of different virus strains and of the same strain after various manipulations. In the course of such work several points bearing directly on the nature of the cell-virus interaction have emerged.

EXPERIMENTAL.

Materials and Methods.

The viruses used were all standard strains that have been frequently described. They were used except where otherwise stated in the form of untreated allantoic fluid. Haemag-

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

glutination methods in general employed 1 p.c. fowl cells the end-point being read from the pattern of deposited cells that had been allowed to settle at room temperature.

Titration of Francis Inhibitor.

Titration of inhibition of haemagglutination by heated LEE virus is based essentially on the method used for antihaemagglutinin titrations in this laboratory but two important modifications must be made in the method. As mentioned in previous papers Anderson (1948) the fowl cells most suitable for antibody titration show practically no inhibition by non-specific mucoids. It is in fact their inability to indicate nonspecific virus inhibition that makes them specially suitable for measuring specific immunological inhibition. Most fowls provide cells suitable for detecting Francis inhibitor and we have used only cells from such birds. In order to avoid the complication of "lipid haemagglutination" the cells are also chosen for absence of this particular susceptibility.

In antibody titrations very little effect is produced by prolonging the time of contact between serum and virus before addition of the red cells. With dilute mucoid solutions there is a relatively slow development to the maximal titre. To a rough approximation 1/4 of the maximal titre is developed in 30 minutes, 1/2 in 120 minutes. Some of the increase in apparent titre after two hours is probably due to weakening of the dilute heated virus. In most respects it would have been desirable to use two hours' contact before addition of the red cells but for the type of experiment being undertaken this made an inconveniently long interval and 30 minutes' contact at room temperature was adopted as the most satisfactory compromise.

The technique used in titrating a given preparation was as follows: Two sets of serial twofold dilutions were made to cover the range within which the end-point could be, normal unbuffered saline being used as diluent. An equal volume (0·25 ml.) was added of LEE virus heated to 56° C. for 30 minutes and diluted in saline to contain 5 A.D's. for the 1 p.c. suspension of fowl cells being used. The mixtures were shaken and left 30 minutes at room temperature before adding a third volume of 1 p.c. fowl cells of the appropriate type. Readings were made by the pattern of sedimented cells as soon as settling was complete at room temperature 20–24° C. Comparative tests had shown that apart from a late inactivation of the virus at pH 6 no significant differences in titre were evident in titrations at pH 6, 7 and 8. The initial mixtures of mucoid and virus were all in buffered saline so that it was not regarded as necessary to use anything but normal saline in the actual titrations.

Purification of Francis Inhibitor from Cyst Mucin.

The great majority of experiments were done with material derived from two ovarian cysts GER and WH_2 while the third was the peritoneal fluid obtained at operation from a case of generalized peritoneal spread of an ovarian tumour. All three were pooled for the preparation of the highly purified mucoid.

"Semipurified mucoid" was prepared from cyst fluid GER by diluting the fluid in two volumes of 5 p.c. NaCl solution adding enough acetate buffer pH 6 to bring the pH just below 7 and boiling for two minutes. Under these circumstances a heavy precipitate of globulins etc. formed leaving a slightly opalescent and straw-coloured supernatant fluid which had a F.I. titre of 10,000 and was used in the experiments as such.

Purified mucoid was prepared as described in a paper by McCrea (1948b). The process in outline was as follows: Two litres of an active cyst fluid were diluted with three litres of normal saline, and acidified with normal acetic acid to pH 5. The fluid was then heated at 100° C. for 15 minutes, cooled, further acetic acid added to pH 4·5, and passed four times through a Sharples centrifuge. After neutralization, the resulting strongly

opalescent solution showed activity of similar order to the original; the total nitrogen was reduced by 94 p.c. (1,340 mg./100 ml. to 80 mg./100 ml.). Four volumes of ethanol were then added to the fluid and, after standing for two hours at room temperature, the precipitate of crude mucoid was removed by filtration, washed with ether, and dried *in vacuo*. The precipitate was taken up in 500 ml. of normal saline and a further amount of denatured protein centrifuged down. The supernatant fluid was then concentrated to small volume *in vacuo* and finally dried *in vacuo*.

The resulting fine brownish powder was ground thoroughly with 90 p.c. phenol, and, after standing for several hours at room temperature, the insoluble fraction was centrifuged down and washed free of phenol with ethanol. The white powder so obtained was found to contain the bulk of the original inhibitory activity. The last traces of non-mucoid protein were then removed by repeated shaking with chloroform-butanol mixture.

The finally purified mucoid was obtained as 1 gm. of white powder, redissolving in distilled water to give an opalescent and slightly viscous solution. No increase in turbidity occurred on adding 10 p.c. trichloracetic acid or on heating to 100° C. The material behaved as a typical mucoid on hydrolysis with normal hydrochloric acid at 100° C., yielding reducing substances and glucosamine. A small proportion of amino acids could also be detected by chromatography. A 1 p.c. solution of the mucoid showed an inhibitory titre of the order of 40,000, which remained constant for at least several weeks at 4° C. in the presence of chloroform. A small amount of insoluble material appeared on storage but removal of this did not influence the inhibitory titre. As a routine the stock solution was diluted 1:8 in normal saline and centrifuged. The supernatant fluid, titre 5,000, was then used to make up the various experimental mixtures to be described.

THE ACTION OF LEE VIRUS ON PURIFIED MUCOID.

The influenza B virus LEE is much more active than any of the other standard strains and most of the detailed experiments have been carried out with it. Allantoic fluid virus harvested not more than five days previously was used. Comparative experiments with dialysed and untreated virus gave no significant differences and since numerous tests with similar preparations of other viruses clearly indicated that no components other than virus or virus derivatives could be responsible for the destruction of inhibitor, untreated allantoic fluid virus was used in all the experiments to be reported.

In most experiments a solution was prepared which after the final addition of virus would contain 0·025 p.c. of purified mucoid in borate buffered saline pH 7·0 containing 0·2 p.c. $CaCl_2$. This was placed in a water bath at 36° C. and at zero time the mixture was completed by adding the appropriate amount of virus. Samples were taken at intervals and the action of virus stopped by immersing the sample for one minute in a boiling water bath. A control tube of mucoid solution without virus was similarly treated remaining in the water bath for the longest period concerned in the experiment. The control F.I. titre in these experiments was approximately 1,000. The actual titre obtained varied somewhat from one experiment to another depending probably on minor variation amongst the cells from different fowls as well as on the accuracy with which the test dilution of heated virus had been prepared. Where necessary the concentrations of mucoid or virus were varied as indicated but the general set up was kept uniform.

(a) *The Influence of Virus Concentration.*

Fig. 1 shows the result of a comparison of five and two agglutinating doses of virus. It will be seen that there is a slight initial lag but that thereafter the rate of destruction

follows a logarithmic course. If time is expressed in hours the constant k of the standard formula $k = \frac{1}{t} \log \frac{A}{A - x}$ is for 2 units 1·1 and for 5 units 2·9. In another experiment in which 0·5, 1, 2, 5 and 10 agglutinating units of virus were compared the values of k were respectively 0·32, 0·64, 1·1, 2·6, 5·8. The value obtained for k with ten agglutinating units of LEE (indicated as k_{10}) was definitely lower in later tests with solutions of the same dried material averaging about 3·4. This is probably to be correlated with the fact that the first solution was made from freshly prepared material which dissolved more readily than subsequent portions which had been kept for some weeks or months in the dry state. Within the limits of accuracy of the titration method and on the assumption that the process is a logarithmic one, it is legitimate to state that the speed of reaction is proportional to the concentration of virus used.

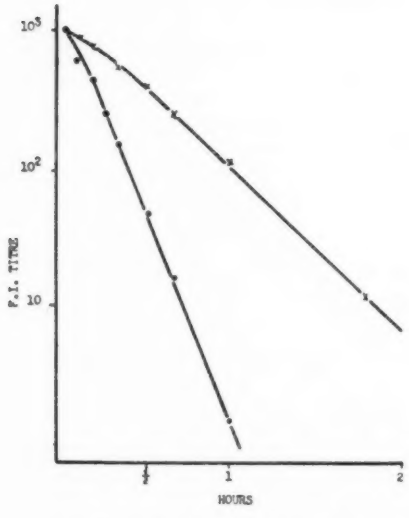

Fig. 1. Action of LEE virus in inactivating Francis inhibitor in purified cyst mucoid at 36° C. pH 7·0.

In this and Figs. 2, 4, 6, 7, 8, 9 and 10 the ordinates represent inhibitory titre against five agglutinating doses of heated LEE virus, using a logarithmic scale. Abscissae: time in hours.

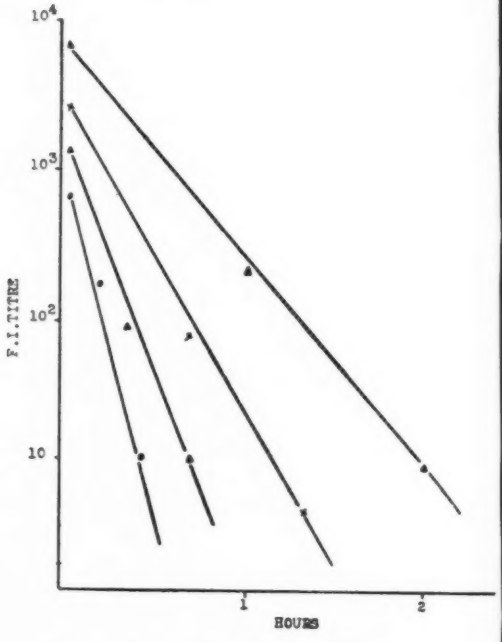

Fig. 2. Action of 10 units of LEE virus on four different concentrations of purified cyst mucoid. Co-ordinates as in Fig. 1.

(b) *The Influence of Substrate Concentration.*

When several different concentrations of mucoid were tested against a standard amount, 10 A.D's., of virus the somewhat unexpected result was obtained which is shown in Fig. 2. The destruction is approximately logarithmic for all substrate concentrations but the slope varies. This seems to indicate that with higher concentrations there is some factor depending on initial concentration but not reduced by virus action, which slows down the rate. The

titration method is not sufficiently accurate nor the end-point sharp enough to allow minor deviations from the logarithmic course to be established.

(c) *Influence of pH and of Ca Ions.*

It was regarded as convenient to use the standard diluent which had been used in all our work with RDE, viz. saline containing 0·1 p.c. calcium chloride and buffered with borate at pH 7·00. Subsequent detailed study of the influence of pH on the reaction indicates that for optimal activity the reaction should be in the neighbourhood of pH 6·2.

Two sets of buffers were prepared to cover the whole range from pH 5 to pH 9 one being McIlwaine's phosphate-citrate buffer in which both anions are Ca deionizing agents and the other a borate-acetate mixture containing throughout 0·1 p.c. $CaCl_2$. Table 1 gives the values of k_{10} with different viruses and for comparison the values of k with twenty units of RDE.

TABLE 1.

Influence of pH and presence of Ca on activity of viruses and RDE

Acetate-borate-Ca					Phosphate-citrate		
pH	LEE	NDV	WSE	RDE	pH	LEE	RDE
4·6	<0·1	..	..	0·33	4·6	<0·1	
5·0	0·30	<0·01	0·023	0·94	5·0	<0·1	<0·1
5·7	5·6	0·07	0·09	2·1	5·5	0·88	..
6·2	>4·6	0·10	0·21	2·3	5·9	0·80	..
6·9	4·44	0·044	0·043	1·6	6·6	1·10	..
7·4	1·32	0·015	<0·02	1·4	6·9	0·84	..
8·0	0·98	<0·01	<0·02	0·54	7·6	0·60	..
8·4	0·64			0·30	8·0	0·48	..
8·9	<0·1			0·22	8·3	0·24	..
					9·1	<0·1	

Fig. 3 presents the results graphically to show the influence of pH and the presence or absence of calcium on the speed of reaction. The point of most interest is probably the difference in Ca ion influence on the virus and on RDE. RDE action is absolutely dependent on the presence of ionized Ca. The action of LEE is greatly reduced but is still greater than that of many weaker viruses in the presence of Ca. It is possible that this signifies the presence of bound Ca within the virus allowing the activity of the virus enzyme even in a medium with no free Ca ions.

(d) *Influence of Temperature.*

This has not been extensively investigated, all standard experiments being carried out at 36° C. The action is very slow at 0° C. as is shown in Fig. 4. In this Figure it will be noticed that there is a fall shown for zero time, i.e. in the tube to which chilled virus was added and which was immediately thereafter placed in a boiling water bath some inactivation of the inhibitor took place presumably during the period of rising temperature. This minimal action of the virus enzyme at 0° C. confirms an earlier unpublished finding of Anderson with Francis inhibitor of serum and is a point on which some importance is laid in the general discussion of the present series of papers.

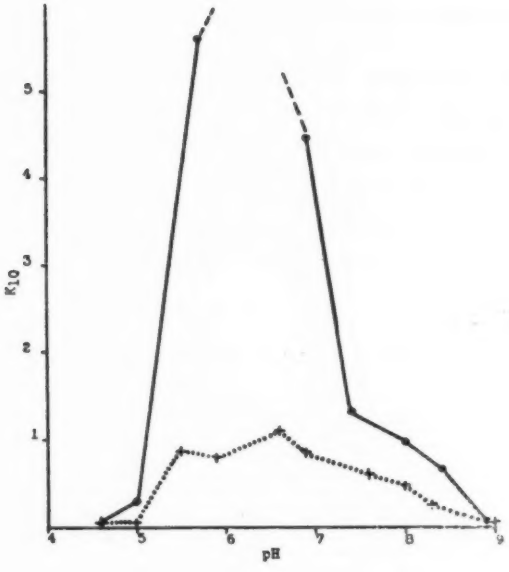

Fig. 3. Influence of pH and presence or absence of Ca ions on activity of LEE virus.

Ordinates: k_{10} against purified cyst mucoid at 36° C. Abscissae: pH. Upper curve in acetate-borate-calcium buffer, lower curve in McIlwaine's phosphate-citrate buffer.

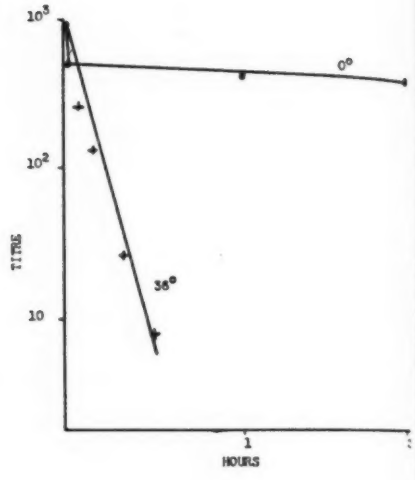

Fig. 4. Comparison of action of LEE virus on Francis inhibitor at 0° C. and 36° C. Co-ordinates as in Fig. 1.

(e) *Use of Partially Purified Virus.*

There is no significant difference in the activity of allantoic fluid virus that has been dialysed through "cellophane" for 24 hours from that of the original material.

Virus was adsorbed on calcium phosphate by Stanley's (1945) method washed in the cold and dissolved with sodium citrate. The solution was dialysed first against tap water and then overnight against distilled water. The haemagglutinin titres were determined and the action against purified cyst mucoid tested by standard technique. Two preparations tested were both considerably less active than the stock virus from which they were derived. The first had k_{10} of 2·5 against 3·4 of the original (in Ca BBS pH 7·0) the second gave k_{10} 1·3 against original 5·1 (at pH 6·2 in acetate borate calcium buffer). In both cases a typical logarithmic curve was obtained.

The cause of the reduced activity of purified virus has not been investigated. Long exposure during dialysis to citrate ion may have affected the intra-virus Ca content which we are inclined to postulate as the reason for the difference in the activity of RDE and virus in Ca free media.

THE THERMAL DESTRUCTION OF ENZYMIC ACTION OF LEE VIRUS.

Throughout recent investigations in this laboratory we have assumed that the loss of the power to elute from red cells which is induced by heating viruses to temperatures between 50 and 55° C. is due to inactivation of the virus enzyme responsible. Briody (1948) has made an extensive study of thermal inactivation of this capacity to elute in different viruses with special reference to the presence of Ca ions and of calcium deionizing agents in the medium.

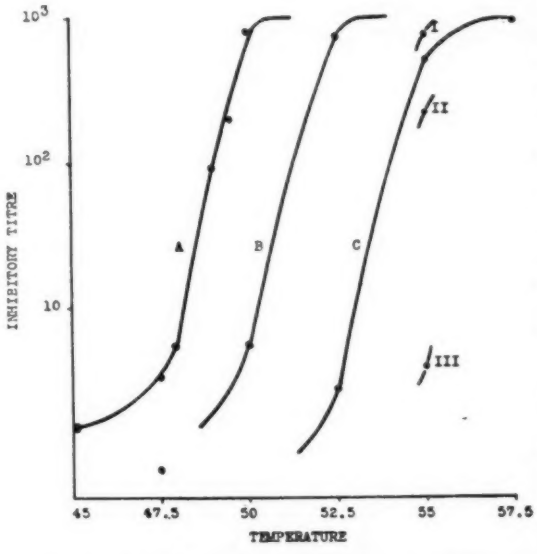

Fig. 5. Development of susceptibility to inhibition by Francis inhibitor, of LEE virus heated for thirty minutes at the temperatures shown under the following conditions:

A. Untreated allantoic fluid pH 7·2. B. Allantoic fluid buffered to M/20 phosphate at pH 7·0. C. Allantoic fluid plus 0·2 p.c. $CaCl_2$ pH 7·0. I. Allantoic fluid plus 0·2 p.c. $CaCl_2$ pH 8·0. II. Allantoic fluid plus 0·2 p.c. $CaCl_2$ pH 7·0. III. Allantoic fluid plus 0·2 p.c. $CaCl_2$ pH 6·0.

Ordinates: inhibitory titre shown with cyst mucoid titre 1,000. Abscissae: temperature.

Burnet and Stone (1947) showed that the cholera vibrio enzyme RDE had a thermal inactivation point approximately 8° C. higher in the presence of excess Ca ions as compared with solutions lacking Ca. It is of interest that a similar effect can be demonstrated on the constituent enzyme of LEE virus.

Virus was used either untreated or with the addition of one-tenth of its volume of appropriate reagents to give the required concentration of phosphate or Ca ions. These were then heated in an accurately controlled water bath for 30 minutes at the temperature shown. The haemagglutinin titre was determined and an appropriate dilution containing five agglutinating units was used to titrate the inhibitory effect of a standard semipurified mucoid solution with a titre of 1,000 when tested with standard heated virus. Each preparation was tested in parallel for its power to destroy the Francis inhibitor of the same mucoid solution under standard conditions.

The results of a number of experiments are depicted in Figs. 5 and 6 and a typical result with untreated virus is shown in Table 2.

No exceptions were found to the rule that when virus had on heating developed the full capacity to act as an indicator of Francis inhibitor, it was wholly lacking in enzymic activity. This correlation also holds when enzymic activity is destroyed by the action of trypsin at 37° C. (Stone, 1948).

TABLE 2.

Effect of heating LEE virus (untreated allantoic fluid pH 7·0)

Treatment	H.A. titre	Francis inhibitor titre	Enzymic activity k_{10}
Unheated	320	<2	2·6
45° C. for 30 minutes	240	2	2·2
47·5° C. for 30 minutes	240	3	2·4
50° C. for 30 minutes	200	800	0·1
52·5° C. for 30 minutes	200	800	0·1

THE ACTIVITY OF DIFFERENT VIRUS STRAINS IN DESTRUCTION OF MUCOID INHIBITOR.

In tests with influenza virus strains other than LEE the same technique was used. Fresh virus preparations usually allantoic fluids were titrated for haemagglutinin activity against 1 p.c. fowl cells and the fluid diluted to give ten agglutinating doses in the final mixture which also contained 0·025 p.c. of mucoid.

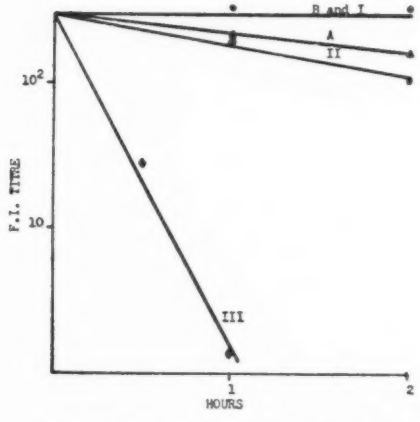

Fig. 6. Enzymic activity of Lee virus heated to 55° C.

A. Untreated allantoic fluid. B. Buffered with phosphate pH 7. I, II, III. With addition of 0·2 p.c. $CaCl_2$ at pH 8, 7 and 6 respectively.

These preparations correspond to those similarly indicated in Fig. 5. Co-ordinates as in Fig. 1.

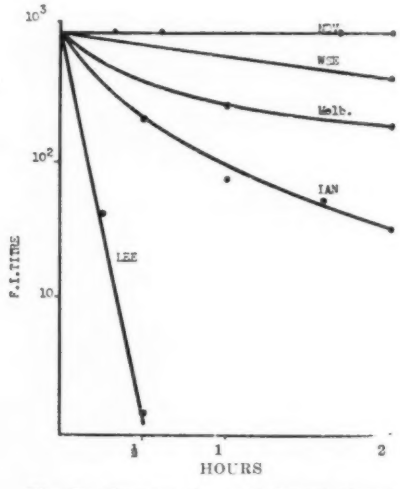

Fig. 7. Comparative activity of viruses NDV, WSE, Melb., IAN-O and LEE on purified cyst mucoid. Each virus 10 agglutinating doses. Co-ordinates as in Fig. 1.

Representative results are shown in Figs. 7, 8 and 9. It is obvious that the experiments would have been more satisfactory if the amount of each virus could have been expressed in micrograms instead of agglutinating units. With the O and ω phase virus fowl cells could not be used and this comparison was made on the basis of ten agglutinating doses with pigeon cells. Our current stocks of LEE give fowl-cell titres which are approximately half those given with human cells. Most of the other strains give approximately equivalent titres with the two types. There is, therefore, a possibility that the superior enzymic activity of LEE may be exaggerated somewhat if the human cell titre is a better index of the actual quantity of the virus than the fowl cell titre.

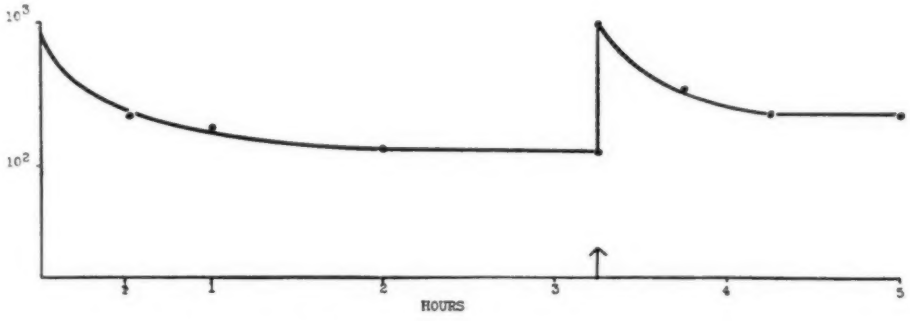

Fig. 8. Action of strain Melbourne on Francis inhibitor in purified cyst mucoid. At the arrow an equal amount of fresh mucoid was added to the system. Co-ordinates as in Fig. 1.

The most interesting finding is the differences between the influenza B viruses LEE and MIL B which follow a logarithmic course going on to complete inactivation of the inhibitor and others such as Melbourne, PR8, and Swine which can take the process only to a certain level. A logarithmic course proceeding to completion is also shown by the cholera vibrio enzyme RDE.

An experiment with the strain Melbourne is depicted in Fig. 8. After the residual F.I. titre had reached an approximately constant level an equivalent amount of fresh mucoid was added to the system. The subsequent course is practically identical with the first part of the curve thus showing that the cessation of action was not due to inactivation of the virus or to the accumulation of products of reaction. The phenomenon is clearly related to the receptor gradient as observed in red cells and like this must depend on heterogeneity of some sort in the population of substrate molecules. The possible nature of the differences amongst the substrate molecules will be discussed later.

In an attempt to obtain further light on the nature of the O-D difference in influenza A strains the enzymic activity of a number of preparations of BEL (Burnet and Stone, 1945) in different phases was tested. Four O preparations from chick embryo lungs all with no trace of agglutinating action on fowl cells at room temperature, an ω embryo lung emulsion with fowl/pigeon cell ratio of 70/800, a δ allantoic fluid resulting from first allantoic passage of an O phase emulsion and stock D phase BEL virus fully adapted to allantoic passage were tested. The dose of virus corresponded in each case to ten agglutinating doses against pigeon cells. All gave similar curves, one for each phase being shown in Fig. 9. Although

from the Figure the order of activity is O > ω̃ = δ > D it is doubtful if the differences are significant. The activity of a more recently isolated strain in the O phase IAN was quite similar to BEL O.

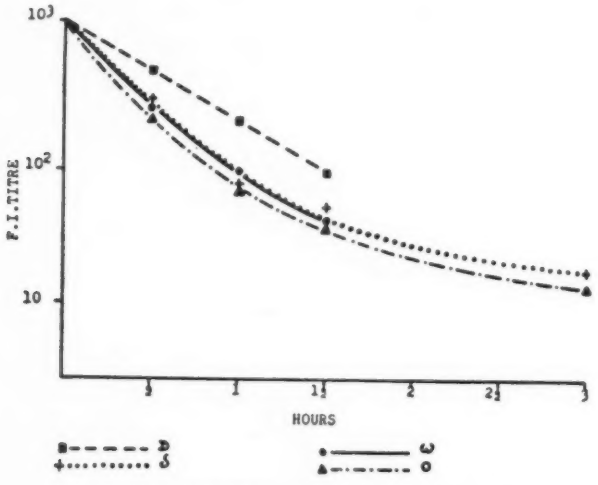

Fig. 9. Action of different phases (O, ω̃, δ and D) of the influenza A strain BEL on Francis inhibitor. Co-ordinates as in Fig. 1.

EXPERIMENTS WITH PURIFIED RED-CELL RECEPTOR SUBSTANCE OF BOVARNICK AND DE BURGH.

Through the kindness of Dr. P. de Burgh we received a small amount of the polysaccharide material isolated from human red cells by de Burgh *et al.*, (1948). In general this closely resembled our purified cyst mucoid in showing its highest inhibitory titre against heated LEE virus and in being rapidly inactivated by RDE and LEE virus.

TABLE 3.

Comparative inhibitory titres of cyst mucoid and red-cell receptor substance

Virus	Cyst mucoid	Red-cell receptor substance
NDV	<1,000	<10,000
PR8	10,000	160,000
Melbourne	<1,000	<10,000
WSE	4,000	1,500,000
H.WSE	2,000,000	1,500,000
LEE	<1,000	<10,000
H.LEE	2,400,000	2,000,000

Inhibitory titre in terms of dry substance against five agglutinating doses of virus using 1 p.c. fowl cells.

The chief difference is that the red-cell substance has a much higher inhibitory titre against active PR8 and WSE than the cyst mucoid. It is to be noted, however, that these two strains are significantly inhibited by the cyst mucoid and that as shown in an accompanying paper (Burnet, 1948b) minimal change by periodate will convert the mucoid to a highly active inhibitor of WSE.

The activity of the red-cell substance is completely destroyed by RDE and by LEE virus. No kinetic experiments were done with RDE but two experiments with LEE showed significantly lower susceptibility to the action of the virus. Using acetate-borate buffer with calcium at pH 6·2 the values of k_{10} were for cyst mucoid 5·1, for red-cell receptor substance 2·3.

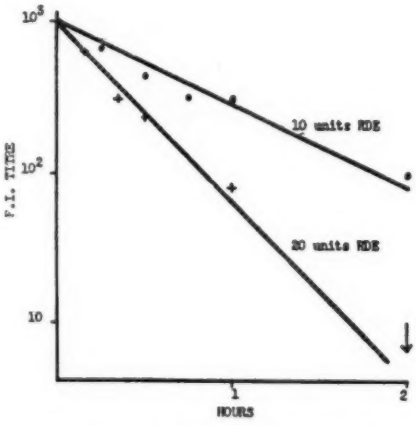

Fig. 10. Action of RDE, at 10 and 20 units, on same purified cyst mucoid used in experiments with viruses. Co-ordinates as in Fig. 1.

THE ACTION OF RDE ON PURIFIED MUCOID.

The course of inactivation of inhibitor by RDE is very similar to that observed with a virus such as LEE. It continues till the inhibitor activity is completely destroyed but in most experiments there is evidence of some slowing up as the reaction proceeds. Fig. 10 shows the result of an experiment in which in place of the standard 10 agglutinating units of virus, 10 and 20 units of RDE as titrated by Burnet and Stone's (1947) method were used. The RDE was in the form of agar culture filtrate which had been heated to 55° C. in the presence of Ca at pH 6 and was subsequently adjusted to neutrality.

As will be seen the inactivation approaches a logarithmic course but there is distinct evidence of slowing down in the later stages. Using the lines shown k has a value of 0·53 with 10 units, 1·1 with 20 units. As the RDE unit has no relation to the haemagglutinin units of virus no useful comparison is possible.

In Table 1 dealing with the effect of Ca and pH on the velocity of reaction the results of parallel experiments with RDE are included. It will be seen that in confirmation of previous work on a less quantitative basis the optimal reaction for the enzyme is around pH 6 and that it has no activity in the absence of Ca ions.

DISCUSSION.

These experiments were undertaken primarily to see whether a clearer picture of the processes concerned in the action of influenza viruses on cell receptors could be gained by the use of a soluble and relatively pure substrate.

The method of estimating the velocity of enzyme action, viz. the estimation of a residual activity by a logarithmic method of titration, does not allow a straightforward analysis of the kinetics of the reaction as developed for systems

in which the amount of substrate altered, or the amount of product obtained, is determined by chemical methods. As far as our results with LEE virus are concerned, the speed of the reaction remains constant throughout the measurable range of concentration and proportional to the concentration of virus. Using a constant virus concentration the course of reaction remains approximately logarithmic, but the slope of the curve is steeper with more dilute concentrations of substrate. In some experiments there was a short lag period before the logarithmic course became apparent, but in all experiments the course over the range from 1,000 to 50 residual F.I. units was logarithmic, the value of k diminishing with the initial concentration of mucoid.

The simplest interpretation of these results is that within the range of concentrations of the reagents used the limit of enzymic capacity of each virus particle is not exceeded. Reaction velocity is determined only by the number of effective collisions of substrate with virus enzyme groups. This will be proportional to the virus concentration and (subject to the increasing effect of viscosity or of other internal interference effects amongst substrate molecules with increasing concentration) to the concentration at any given moment of active F.I. molecules. A subsidiary postulate is that inactivated molecules have no reactivity with the virus enzyme, but that their capacity to give rise to viscosity or similar types of internal interference is unaltered by the enzymic action. Since no change in viscosity in mucins acted on by RDE is detectable (Burnet, 1948a), this is in accord with the evidence.

The fact that some viruses can carry the destruction of the F.I. of purified mucoid only to a certain level, while others, notably LEE, proceed logarithmically to complete inactivation, appears to be closely related to the phenomena concerned in the receptor gradient.

Two possibilities present themselves: (1) The F.I. titre of a given preparation is a measure of the number of unmodified molecules present. Under the action of virus or RDE the active molecule is converted by a single change into an inactive molecule. When virus Melbourne produces about a 90 p.c. reduction in F.I. activity and then virtually ceases to act, it must be assumed that this 90 p.c. of active molecules differ from the remaining 10 p.c. in having a configuration allowing effective adsorptive or other type of union with the virus and so allowing enzymic activity. Strain LEE is more readily adsorbed and is effective against all the minor variants in the molecular pattern of F.I. present in the population of molecules. (2) F.I. titre is a more complex resultant of change within large polymerized molecules on microaggregates of the mucoid. We may assume that each unit has on the average M points at which enzyme may act and that with each action the resulting units with M–1, M–2 etc. points have lowered adsorptive power for virus. This results both in lowered F.I. titre and in an apparent diminution of enzyme activity by any of the active viruses. On this view the situation when Melbourne virus ceases to act is that there are

still the same number of mucoid units present, but their average number of susceptible points is M–N. This has reduced the adsorptive affinity for virus Melbourne to such a low level that further enzymic action by that virus is virtually impossible. With LEE on the other hand the adsorptive affinity and the velocity of reaction are reduced only in the ratio $\frac{M-N}{M}$, so that the reaction continues along a logarithmic course.

On the whole the second alternative seems more likely, mainly because such a concept can be more readily transferred to the conditions on the cell surface. Much more detailed physicochemical study of the substrate solutions is obviously called for before any satisfactory interpretation is possible. The significance of the findings reported in this paper to the general problems of virus-cell interaction are discussed in the final paper of this series (Anderson *et al.*, 1948).

SUMMARY.

Using a purified ovarian cyst mucoid as inhibitor of haemagglutination by heated influenza B virus (Francis inhibitor), a study has been made of its inactivation by influenza and related viruses.

With the more active viruses, exemplified by LEE (B), the inactivation follows an approximately logarithmic course, the rate being directly proportional to the virus concentration.

The rate of inactivation is highest at pH 6·2 and falls off rapidly below pH 5·7 and above pH 7·0. It is much higher in the presence of free Ca ions than in their absence.

Inactivation proceeds much more rapidly at 36° C. than at 0° C.

With LEE virus the temperature at which enzymic activity is destroyed depends on the medium in which it is suspended. Loss of enzymic activity is directly correlated with development of capacity of the virus to act as an indicator of Francis inhibitor.

With less active viruses the inactivation of Francis inhibitor proceeds only to a certain level. This is due not to inactivation of the virus enzyme, but to the exhaustion of that proportion of the substrate susceptible to the action of the virus concerned. The relation of this phenomenon to the "receptor gradient" is discussed.

There are no significant differences in the enzymic activity of O and D phases of an influenza A strain.

The "receptor substance," isolated by Bovarnick and de Burgh from human red-cells, behaves essentially similarly to purified cyst mucoid in its susceptibility to inactivation by viruses and the vibrio enzyme RDE.

Acknowledgment. I am indebted to Mr. J. F. McCrea for the preparation of cyst mucoid, to Dr. P. de Burgh for the red-cell receptor substance and to Miss Joan Melville and Miss Marie McPherson for their help in the large number of inhibitor titrations required in this work.

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MUCINS AND MUCOIDS IN RELATION TO INFLUENZA VIRUS ACTION

VI. GENERAL DISCUSSION

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Preceding papers of this series have been concerned with experimental studies of the interaction between viruses of the influenza group and the "receptors" of both red-cells and the susceptible cells of the allantoic cavity or the mouse lung. It has also been shown that the "Francis inhibitor" of serum and of various mucinous secretions is closely related to the cell-receptor substance in that its reactivity with heated LEE virus is destroyed in similar fashion by the enzyme RDE and by influenza viruses.

It has been thought convenient to confine discussion in each of these papers to points specifically arising in their substance and in the present paper to submit a general discussion of the nature of the reactions involved.

A general account of the work leading up to these investigations has already been published (Burnet, 1948a) and in that paper it was concluded that the influenza viruses are adsorbed by all receptors, the essential component of which was a mucopolysaccharide. Following Hirst (1943) it was also considered probable that enzymic destruction of the receptor by the virus was a necessary preliminary to infection.

The most contentious part of this interpretation is the assumption that influenza viruses carry, as part of their surface structure, enzymes or enzymatically active configurations which are essentially similar to the soluble enzyme from *V. cholerae* which we have referred to as RDE (receptor-destroying enzyme) and act similarly on the same type of substrate. Up to the present it has proved impossible to define what aspect of the substrate mucoid is involved in the reaction or to isolate chemically any products of the reaction of either virus or RDE with mucoid. All the evidence of enzymic activity is derived from biological rather than chemical tests. If an enzyme is defined as an agent producing or accelerating a specific chemical change in a substrate of known character, no proof of the enzymic nature of either agent has been obtained. A

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broader interpretation of the term enzyme is, however, usually accepted to cover circumstances in which quantitative changes in the substrate can be measured only by biological tests. In practice such an interpretation has usually been adopted until advancing knowledge has made it possible to define either the nature of the enzyme or the details of its action on the substrate. Our use of the word enzyme in what follows is throughout subject to this qualification.

The Correspondence Between Virus Enzymes and RDE.

The probable existence of an enzyme in the surface structure of the influenza virus was explicitly recognized by Hirst (1942) in his work on the phenomenon of elution of virus from red-cells. The fact that a given virus preparation can react with red-cells rendering them incapable of further virus adsorption and after release produce the same series of effects on further amounts of red-cells is hardly susceptible of any other interpretation. We have been concerned to establish first, that in all essential respects the activity of the virus enzyme can be paralleled by the enzyme RDE, produced by cholera vibrio strains, and second that the substrate of both enzymes was associated with, or represented a particular component of, a variety of mucopolysaccharide complexes.

RDE has not yet been obtained in pure form. The assumption that all the activities we have ascribed to RDE are in fact the properties of a single enzyme must therefore be based on the following indirect considerations: (1) All essential experiments have been carried out with two types of RDE preparation, (a) heated to 55° C. in the presence of excess calcium ions at pH 6 and (b) concentrated by adsorption and elution from red-cells. In all respects the two types of preparation show activities proportional to their titres as assessed by our standard method. This involves the determination of the limiting dilution of enzyme, which acting on red-cells under standard conditions produces partial loss of agglutinability by virus. (2) All the activities described are inhibited by an antiserum active against RDE (Burnet, 1948c) and not by other antisera prepared against similar cholera filtrates but containing no anti-RDE. (3) The internal consistency of the results and their equivalence to activities of virus in themselves strongly support the view that the activities ascribed to RDE are in fact those of a single enzyme.

The correspondence of the action of viruses and RDE on red-cells has been shown in detail by Stone (1947), and in the present series of papers further correspondence in their action on mucins and mucoids is described. In work at present only partly published, Whitten (1947) has shown that both RDE and LEE virus rapidly and completely destroy the biological activity of gonadotrophin (prepared from pregnant mare serum).

The results are consistent with the view that the enzymes concerned affect the same substrate and by their action produce the same changes in biological activities of the substrates. The possibility must remain, however, that the two

types of enzyme differ in the means by which they alter or destroy the same biologically active configurations. Detailed work at the chemical rather than at the biological level will be needed to determine this.

The enzymic activity of both RDE and LEE virus is dependent on the presence of Ca ions and the optimal reaction (about pH 6·2) is the same. The conditions of thermal inactivation of RDE and virus enzymes also point to a basic similarity between the agents. This similarity was first observed by Briody (1948), using the capacity of virus to elute from red-cells as a measure of enzymic activity. More detailed work using the inactivation of Francis inhibitor as index is presented in Paper V of this series. In the absence of Ca ions and in an alkaline medium the enzymes in both cases are inactivated at lower temperatures than in the presence of Ca or at a pH below 7. The haemagglutinating capacity of the virus persists to a considerably higher temperature than is required to destroy the enzyme. The significance of this dissociation between haemagglutinating and enzymic activities is discussed later.

Substrates of Virus and RDE.

Studies of the various physiological and pathological materials in which the substrate (as identified (a) by its power to inhibit haemagglutination by heated LEE virus and (b) by the destruction of that power with RDE) is present have so far been at a rather superficial level and any conclusions must be tentative. McCrea (Paper II) has provided evidence that the Francis inhibitor in normal human or rabbit serum is a mucoid. The titre observed varies considerably from one individual to another and from species to species. All human glandular secretions which contain true mucin, as defined by a positive ACRA test (Burnet, 1948a, 1948b) and the production of a characteristic fibrous precipitate with acetic acid or CTAB, contain the inhibitor in amounts closely correlated with the mucin content. The richest sources so far found are cervical mucus and the contents of certain ovarian cysts. The type of ovarian cyst producing high titre material is at present under study. In contrast to our first impression, typical pseudomucinous cysts are usually poor producers. The highest yield was from two cysts that contained a considerable proportion of true mucin. Large amounts were also present in serous type cysts containing a high concentration of coagulable protein, and it was from cysts of this type that the purified mucoid used for the work described in Paper V was prepared. Purified blood group substance was active, but to lower titre and material from swine stomachs quite inactive. It is clear that there is no necessary correlation between blood group activity and the Francis inhibitor quality. The relationship between the two activities remains to be determined. As a working hypothesis we consider it likely that a considerable range of mucin or mucoid molecules may carry one or both of the configurations responsible for blood group specificity and for the Francis inhibitor character respectively. The very high activity of

two purified mucoids, Morgan's human blood group O substance and that prepared by McCrea (to be published), is the only direct evidence that the substrate of RDE and of the virus-enzymes is of mucopolysaccharide nature. A wide variety of indirect evidence accords with this conclusion.

Very little work has yet been done to determine the distribution of Francis inhibitor in different animal species. All mucous secretions from human beings appear to contain it in rough proportion to the content of mucin. Guinea-pig mucin is also active, but bovine cervical mucus has no activity nor has any been shown in gastric mucus from the pig.

A number of papers have recently appeared concerning the inhibitory activity of certain polysaccharides on virus haemagglutination or infectivity. Green and Woolley (1947) found that a number of polysaccharides including blood group substance A would inhibit haemagglutination by influenza A virus. Apple and citrus pectins were in addition shown to have some power of preventing infection when used in high concentration in the allantoic cavity. Ginsberg, Goebel and Horsfall (1947) have shown that mumps virus haemagglutination is inhibited by Friedländer bacillus polysaccharide and its power to infect the allantoic cavity greatly reduced. No evidence has yet been produced that the inhibitory effect of these polysaccharides is destroyed by the viruses or by enzymes of RDE type. Until this is available the inhibition observed must be ascribed to the adsorption of the polysaccharide by the virus perhaps depending on significant similarities to the polysaccharide of the cell receptors.

Of more direct interest to the present discussion is the isolation from human erythrocytes by Bovarnick and de Burgh (1947, 1948) of a polysaccharide that inhibits strain PR8. By extraction with lipid solvents they obtained, in the first instance, material capable of inhibiting haemagglutination by mumps and PR8 viruses. Further fractionation allowed the separation of the inhibitor from the lipid complex in the form of a polysaccharide not yet characterized in detail. They observed partial destruction of the inhibitor activity by prolonged contact with virus and recognized the similarity of this action to that involved in the elution of virus from red-cells. With a small sample of Bovarnick and de Burgh's polysaccharide we have been able to show that its biological activities very closely resemble those of our purified cyst mucoid and that they are similarly destroyed by RDE and LEE virus.

There is little evidence as to the mode of attack of virus enzymes or RDE on the substrate. Following Hirst's brief statement of the effect of periodate on the red-cell receptor, we have shown that various types of Francis inhibitor are rapidly inactivated by small amounts of this reagent. This suggests that the inhibitor activity is dependent on the structure of the polysaccharide, but the possibility that a peptide component is also concerned has by no means been excluded. The chemical nature of the mucoids and of the action of RDE is at present under active investigation in this laboratory.

The Interaction Between Virus and Cell-Receptor.

As Anderson (1947) has recently pointed out, the existence of an active enzyme on the virus particles does not in itself provide an explanation of the phenomenon of haemagglutination and still less of the initial phase of infection in susceptible cells. There are two well-defined circumstances in which agglutination occurs with full activity but no enzyme action takes place: these are (1) when virus heated to 55° C., particularly influenza B strains, agglutinates untreated red-cells and (2) when active virus agglutinates cells treated with an appropriate amount of periodate (Fazekas de St.Groth, 1948a, 1948b). And in the opposite direction it can be shown that an influenza A virus in the O phase with no power to agglutinate or be adsorbed by fowl cells is as active as the corresponding D strain in enzymatically destroying the Francis inhibitor of purified mucoid.

Anderson considered that the best representation of the conditions is to state that effective adsorption to the substrate is a necessary pre-requisite for enzyme action, but that adsorption is partly or wholly a function of components of the virus surface other than the active enzyme groups. This does not preclude the possibility that the adsorptive force betwen virus and cell "receptor" is influenced by the conformation of the enzymic groups.

The speed of destruction of purified Francis inhibitor by virus *in vitro* is so high that it is almost inconceivable that virus is held to red-cell receptor by a single enzyme-substrate union. If this were so, elution would be a much more rapid process than it is, and it would also be almost impossible to provide any explanation for the existence of a receptor gradient.

It has been essential for the development of our work to have some form of working hypothesis as to the nature of cell receptors and the interaction of the virus particles therewith. The following picture of the conditions appears to us to be in accord with the facts so far obtained: the "receptors" on the red-cell are areas made up predominantly of mucopolysaccharide molecules which are presumably built into the lipid-protein mosaic of the cell surface. In any given patch there are many hundreds or thousands of the particular atomic configuration of the substrate which is changed by the action of virus-enzyme or RDE. With progressive action of the enzyme these susceptible configurations diminish in number and concomitantly the adsorptive affinity of the patch for viruses of the influenza group diminishes.

It is hard to believe that the adsorptive power of the receptor for virus and its susceptibility to inactivation by the virus enzyme are not closely related, but our experiments have given no positive evidence to support such a view. All that can be said is that by the action of any of the effective enzymes the receptors become progressively incapable of adsorbing an increasing number of viruses, so giving the phenomena of the receptor gradient. When a virus

such as the Melbourne strain of influenza A is adsorbed to a receptor area one, or more probably several of its surface enzymes are in a position to act on appropriate substrate configurations. As each unit of enzymic action is completed, the enzyme concerned is freed for action on an adjacent susceptible configuration. Under the action of Brownian movement and the various adsorptive forces concerned, the virus particle can be regarded as "browsing" over the patch until such a condition is reached that the adsorptive forces are no longer strong enough to maintain it in contact with the receptor. When the action of strain Melbourne has ceased, there are still many available substrate configurations, but only viruses more readily adsorbed than Melbourne are capable of further reducing their number.

With two such complex surfaces as those of the virus particle and red-cell, it is highly probable that there are qualitative differences which may be superimposed on the quantitative differences in adsorptive affinity revealed in the receptor gradient. There is already some evidence that mumps virus differs considerably from the rest of the group (Bovarnick and de Burgh, 1947; Stone, 1947). In addition, tests with cell types such as ox and horse show only certain viruses capable of producing haemagglutination and considerable differences from fowl on human cells in the order of the receptor gradient.

It is naturally impossible to carry out equally detailed experiments with cells susceptible to infection by the virus, but as far as evidence is available, the conditions are essentially similar to those obtaining with erythrocytes (Hirst, 1943; Fazekas de St.Groth, 1948a). Stone's (1948a, 1948b) experiments on the preventive effect of RDE treatment of the allantoic cavity, however, indicate that the intensity of the effect is not related simply to the position of the virus concerned in the receptor gradient, as determined with fowl red-cells. It may be that adsorption to the lining cells of the allantoic cavity is mediated by a mucopolysaccharide which differs from that of the fowl erythrocyte and that if the experiments were made a different order of viruses in the receptor gradient would be obtained. The Henles' (1947) work on the sequence of destruction of various activities of influenza virus by ultraviolet irradiation also indicates a dissociation of the capacities (a) to agglutinate and elute from red-cells and (b) to cause interference in the allantoic cavity with infection by active virus. It seems certain that the virus-receptor system in which we are interested is only one portion of the complex mechanisms which determine the result of contacts between virus and susceptible cells.

The Significance of the Francis Inhibitor Phenomenon.

The essence of Francis' discovery (1947) was that heated influenza B virus was inhibited to high titre by normal serum while the same virus unheated was inhibited only to a trivial titre or not at all. The significance of this difference requires some consideration.

The relevant facts are: (1) The phenomenon is shown by all influenza B strains with one exception (Francis). We have found in addition that the strain WSE on heating becomes almost as sensitive an indicator as heated B strains and that our strain of Swine 15 also becomes much more susceptible to inhibition on heating, though the titres obtained are much lower than those with heated B strains. Other A strains, mumps virus and N.D.V., tested either by Francis or ourselves, do not show the change in character on heating.

(2) All influenza and N.D. virus strains heated between 52° C. and 55° C. fail to elute from red-cells to which they have been adsorbed, irrespective of whether or not they showed increased susceptibility to inhibition by mucoids. As far as they have been tested, they also show absence of enzymic action on suitable substrates. In other words, destruction of enzymic activity with retention of adsorptive and agglutinating power does not necessarily make the virus susceptible to inhibition by appropriate mucins or mucoids.

(3) When strain LEE is heated under various conditions, the capacity to act enzymically on Francis inhibitor is lost and power to function as an indicator of Francis inhibitor is gained at the same temperature levels. In addition, LEE virus treated with trypsin at 37° C. showed a loss of enzymic activity with concomitant development of susceptibility to inhibition by mucoids (Stone, in press). The correspondence between the two types of change in our experiments was complete.

(4) The full titre of Francis inhibitor is only shown when suitable red-cells are used; any human cells will serve, but cells from a minority of fowls show practically no inhibition of haemagglutination under circumstances in which other fowl (or human) cells demonstrate a full inhibitory effect.

(5) Francis inhibitor titrations require an appreciable period of contact between inhibitor solution and heated virus before red-cells are added, if the full titre is to be obtained. Titrations can be made either in the refrigerator or at room temperature and the difference between heated and unheated virus is equally evident at either temperature.

These facts appear to exclude any simple hypothesis that destruction of enzymic activity by heat or by other means is in itself sufficient to make the virus an effective indicator of Francis inhibitor. The fact that the apparent inhibitory titre of a given serum or mucoid solution against heated LEE virus differs greatly according to the type of red-cells used as indicator, shows at once that a dynamic adsorptive equilibrium is involved.

In the system, inhibitor-virus-red-cell, adsorption of virus with consequent haemagglutination will occur only when the virus particles are insufficiently blocked by adsorbed inhibitor. Essentially two adsorptive equilibria are involved—inhibitor to virus and virus to red-cell surface. There is a "competition" for the virus between mucoid and cell surface, and the result will depend essentially on the relative "affinity" of the competitors for the virus particles.

The affinity of red-cells for viruses differs according to the source of the cells, and for the easy detection of inhibitors other than antibody, cells of relatively low affinity for virus must be used. The affinity between inhibitor and virus will naturally depend on both components, and treatment of either may modify it. In Paper IV of this series it has been shown that minimal treatment of cyst mucoid with periodate will greatly increase its blocking activity for unheated WSE virus, while heating of WSE virus greatly increases the inhibitory titre of untreated mucoid against its haemagglutinating action.

Where the virus is enzymically active the adsorption equilibrium with a susceptible mucoid will be modified according to the activity of destruction of the mucoid. However, Anderson's finding (Paper I) that the difference between heated and unheated LEE as indicators of Francis inhibitor is practically the same at 4° C. (when enzymic action is slow) as at room temperature, indicates that this factor is not of significant importance under the usual conditions of the experiments *in vitro*.

Our conclusion then is that on heating a virus such as LEE to 55° C., changes in its surface configuration are produced which increase its adsorptive affinity for various mucoids. It is tempting to think that in the case of this virus at least the changes which destroy its enzymic activity are concomitantly and directly responsible for the changed adsorptive affinity. Since, however, no such changes can be detected in strains like NDV and Melbourne, any such suggestion cannot be generalized.

GENERAL CONCLUSION.

We have presented the evidence that unless certain mucopolysaccharide molecules are present in the surface of the cell, viruses of the influenza group can neither agglutinate red-cells, nor adsorb to, nor infect susceptible cells. We have also shown that the viruses can actively destroy the biological activity of these cellular polysaccharides and of similar mucopolysaccharides or mucoproteins in solution. It has not been possible to show what part this enzymic action of the virus plays in the process of infection. One possible hypothesis is that a firm adsorption to the surface of the cell is needed to allow entry of virus into the cytoplasm and that subsequent to entry the further processes leading to multiplication of the virus cannot proceed until the virus has freed itself from the adsorbed mucoid.

From the practical point of view these investigations are only significant if they offer some approach to the prevention or effective treatment of the diseases produced by viruses of the group. Theoretically there are several possible ways in which a prophylactic or therapeutic effect could be developed on the basis of these conceptions. (1) Cell receptors in the respiratory tract might be removed by regular inhalation of RDE or other destructive agent. (2) An effective "competitive poison" for the virus enzyme might be similarly

administered which, when deposited on the mucous film lining the respiratory tract would render this an effective barrier against infection, both initial infection from without and the spreading surface infection of the mucosa which follows the initiation of infection.

Neither of these approaches seems even remotely practicable. As has been pointed out (Editorial in *Lancet*, 1948), the action desired would take place at the surface of the cell and there is already available in the form of antibody a theoretically much more effective agent for dealing with virus outside or on the surface of the cell. Neither experimentally nor clinically has inhalation of atomized antiserum proved a practical means of aborting or treating influenza. (3) An approach perhaps more worthy of consideration is to exploit the idea that the normal respiratory mucous film is an important natural defence against viruses of this group. If so, anything that increased the effectiveness of this film, either by maintaining its coherence or within limits increasing its thickness, would render more unlikely infection by any virus actively adsorbed by mucin. We wonder, for instance, whether there is any evidence that persons with colds are more or less likely to contract influenza than similarly situated individuals without colds. Or whether the much lower incidence and mortality of pandemic influenza in the middle-aged and elderly was due to their greater mucous secretion in the respiratory passages rather than to immunological factors.

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THE TOTAL PHOSPHORUS, PHYTATE PHOSPHORUS AND INORGANIC PHOSPHORUS CONTENT OF WHEAT, AND ITS MILL PRODUCTS

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The total phosphorus content of cereal grains and of bran and pollard is normally high compared with most foodstuffs. A high but variable proportion of this phosphorus occurs as phytin, a calcium, magnesium potassium salt of inositol-hexa-phosphoric acid or phytic acid. Considerable evidence exists that phytic acid is not readily available to non-ruminant species as a source of phosphorus. Sheep, and presumably other ruminants, can completely hydrolyse phytic acid and probably utilize the liberated phosphoric acid (Reid, Franklin and Hallsworth, 1947). Phytic acid can also interfere with calcium absorption, due to its capacity to form insoluble calcium phytate in the intestine, although recent evidence (Walker, Irving and Fox, 1946) suggests that high phytate diets do not necessarily induce permanent negative calcium balances in humans.

In Australia, interest in the phosphorus fractions of wheat and its milling products is related both to their capacity to supply available phosphorus and to possible influences on calcium absorption. Pigs and poultry, which have high calcium and phosphorus requirements, are fed very largely on wheat, bran and pollard. White flour, in the form of bread and other foods, forms an important part of the diet of the Australian population. Nutritional surveys carried out in 1936–7 and 1944–5 disclosed the fact that considerable numbers of people were consuming less than the "recommended" amounts of calcium for nutritional adequacy. Data on the phytic acid content of white flour, and other cereal products consumed by humans, are of value therefore in helping to throw light on one of the factors influencing the availability of the calcium which is consumed.

Underwood and Snook (1935) have shown that Western Australian wheat bran and pollard are very much lower in total phosphorus than average English or American wheat and its milling products, as judged by "standard" analyses. Dadswell (1935) also showed that Victorian wheat was appreciably lower in this constituent than average overseas samples. Snook (1938) confirmed this finding for Western Australian wheat and showed further that the phytic acid

[1] Working under a grant from the National Health and Medical Research Council.

phosphorus content was correspondingly low. Burch (1946) obtained results for Western Australian wheat very similar to those of Snook (*loc. cit.*). No Australian analyses of bran, pollard and flour for phytic acid phosphorus appear to have been published, apart from a few figures given in the Annual Report of the Government Analyst, Queensland (1946). No determinations of the inorganic phosphorus content of Australian wheat or its milling products have been published, so far as is known.

EXPERIMENTAL.

Sampling.

Samples of wheat, bran, pollard and flour were obtained in March, July and September, 1947, from each of four mills widely separated in Western Australia. On each occasion 2 lb. samples were obtained by using four half-pound sub-samples collected at half-hour intervals during the running of the mill. The milling products were drawn from the millstreams shortly after the wheat was collected in each case. In this way the bran, pollard and flour were, as far as possible, representative of the wheat sampled in each mill and the samples as a whole could be taken as a reasonable representation of the wheat and its milling products available for consumption in W.A. during 1947. All wheat and bran samples were ground to pass a 30-mesh sieve prior to analysis.

Extraction Percentages.

These were obtained for only seven of the twelve sets of samples. The variation, however, was small and averaged as follows: bran 15 p.c., pollard 13 p.c., flour 72 p.c.

Methods of Analysis.

Total phosphorus. For the determination of total phosphorus the wet digestion method of Piper (1939) was used, followed by colorimetric estimation by the method of Fiske and Subbarow (1925). The reproducibility was found to be $\pm$ 0·5 p.c.

Phytic acid phosphorus. The methods of McCance and Widdowson (1935), Pringle and Moran (1942) and Earley (1944) were investigated. The method of McCance and Widdowson was found the most satisfactory and gave results, reproducible to within $\pm$ 0·5 p.c., when the colorimetric phosphate estimation method of Fiske and Subbarow (1925) was used.

Inorganic phosphorus. The method of Berenblum and Chain (1938) modified by Pons and Guthrie (1946) was adopted, except that the phosphomolybdate was extracted with n-butanol instead of iso-butanol. This small modification proved quite satisfactory and the results were found to be reproducible to within $\pm$ 3 p.c.

RESULTS.

The mean total phosphorus and phytate phosphorus contents of the twelve sets of samples expressed as percentages on the dry basis, together with the mean percentage of the total phosphorus present as phytate phosphorus and the coefficient of variation for each material, are given in Table 1. The overall variability is small except in the case of the phytate phosphorus content of the pollard samples. It can be stated further that there were no consistent differences between the four mills or between the same mills sampled at different times. The mean values presented can, therefore, be accepted with confidence.

TABLE 1.

Total and phytate phosphorus of wheat and mill products. Percentages on dry basis.

Material.	Percentage extraction.	Total phosphorus.		Phytate phosphorus.		Phytate P as percentage of total P.	
		Mean percentage.	Coefficient variation.	Mean percentage.	Coefficient variation.	Mean percentage.	Coefficient variation.
Wheat	—	0·256±·006	8·4	0·172±·005	9·7	67·1±0·6	3·3
Bran	15	0·914±·032	11·9	0·751±·028	12·8	82·4±1·4	5·7
Pollard	13	0·471±·018	13·0	0·334±·019	19·4	70·4±1·8	8·7
Flour	72	0·090±·002	8·9	0·026±·001	12·3	29·4±0·9	10·3

As a check on the results, the total phosphorus and phytate phosphorus contributed by the combined wheat fractions were compared with the amounts present in the wheat, using the average extraction figures for bran, pollard and flour presented earlier. The results showed a very satisfactory agreement. The recovery of total phosphorus calculated in this way was 102·2 p.c. and of phytate phosphorus 96·4 p.c. For the seven sets of samples, for which actual extraction percentages were obtained, the recovery was very close to 100 p.c. in each case.

Inorganic phosphorus determinations were carried out on only one set of samples from each mill. The results, expressed as percentages on the dry basis, together with the proportion of total phosphorus present as inorganic phosphorus, are given in Table 2.

TABLE 2.

Inorganic phosphorus content of wheat and mill products. Percentage on dry basis.

Material.	Inorganic phosphorus.		Inorganic P as percentage of Total P.	
	Mean p.c.	Range p.c.	Mean.	Range.
Wheat	0·012	·011—·013	4·5	4·2—4·9
Bran	0·039	·031—·048	4·5	4·1—5·2
Pollard	0·025	·022—·028	6·1	5·5—7·0
Flour	0·004	·004—·005	4·7	4·4—5·5

DISCUSSION.

The total phosphorus figures given for wheat and pollard in Table 1 do not differ greatly from the average values for these materials reported by Underwood and Snook (1935). The figure for bran in this Table is, however, appreciably higher than that given in the earlier paper. Snook (1938) and Burch (1946) also found values for the total phosphorus of Western Australian wheat which agree closely with those of Table 1. In all these investigations the total phosphorus levels are substantially lower than most overseas analyses. Thus Morrison (1937) gives the following figures for the total phosphorus content of "average" American wheat, bran, pollard (middlings) and white flour:

0·43, 1·32, 0·94 and 0·13 p.c. on the dry basis. "Standard" English analyses taken from the Ministry of Agriculture Bulletin, No. 48 (1933), are 0·43, 1·42 and 1·32 p.c. (dry basis) for wheat, bran and pollard (middlings) respectively. The difference between these figures and those obtained in the present investigation and in the earlier one of Underwood and Snook (1935) can readily be seen by inspection of Table 3.

TABLE 3.

Comparison of total phosphorus content of "Standard" English, American and Western Australian wheat and mill products.

Percentages on the dry basis.

Source.	Authority.	Wheat.	Bran.	Pollard.	Flour.
U.S.A. ("Standard")	Morrison (1937)	0·43	1·32	0·94	0·13
Great Britain ("Standard")	Min. of Agric. Bull. 48 (1933)	0·43	1·42	1·32	0·12*
Western Australia	Underwood and Snook (1935)	0·23	0·67	0·40	—
Western Australia	present investigation	0·26	0·91	0·47	0·09

* Pringle and Moran (1942) (70 p.c. extraction).

Evidence from other investigations in general support this finding. For instance, Pringle and Moran (1942) studied the total and phytate phosphorus of wheat and mill products from a number of sources. They report the total phosphorus content of Manitoba wheat to be 0·38 p.c., Plate wheat 0·48 p.c. and English wheat 0·45 p.c. on the dry basis. Feyte (1933) found the total phosphorus of French wheat to range from 0·35 to 0·62 p.c., but 79 p.c. of the samples contained between 0·42 and 0·53 p.c. Syrian wheat, on the other hand, was found by this worker to contain only 0·26 to 0·31 p.c., a level very little higher than that found in the present investigation.

The proportions of the total phosphorus present as phytic acid, as shown in Table 1, tend to be slightly lower than most of the values reported by overseas workers for similar materials, but the differences are extremely small and not in any way comparable with the differences mentioned in the previous paragraph. Consequently, the relatively low total phosphorus levels are paralleled by almost equally low levels for phytic acid phosphorus, compared as before with most overseas figures.

The inorganic phosphorus values presented in Table 2 are of interest in showing whether there is any disproportionate reduction in this fraction. Few figures are available for comparison, but the mean proportion of inorganic to

total phosphorus in wheat (4·5 p.c.) is very similar to the figure 4·9 p.c. given by Webster (1928) for an American wheat of very much higher total phosphorus content, and by Urazownicka (1933) for Polish wheat, namely 5·0 p.c.

It is not proposed to discuss the nutritional implications of the above findings in this paper, nor to speculate at this stage as to the factors responsible for the low phosphorus values. The latter will form the subject of further investigation. Nor is it known whether wheat and mill products from other parts of Australia are similar in phosphorus content to Western Australian products or conform more to overseas values. Evidence on this point is exceedingly meagre. Dadswell (1935) gives figures for Victorian f.a.q. wheat about 30 p.c. higher than those reported in Table 1, but still appreciably below the "Standard" English and American values cited above. Burch (1946) analyzed three N.S.W. white flours for phytic acid phosphorus and found the following values expressed on the dry basis: 0·032, 0·040 and 0·046 p.c. These values compare more closely with the figures given for English 70 p.c. extraction flour by Widdowson (1941) (0·051 p.c.) and by Pringle and Moran (1942) (0·038 p.c.) than those found in the present investigation (mean, 0·026 p.c.). No proper assessment of the quantitative importance of phytic acid in the nutrition of the Australian people can be made until more data become available for wheat, flour and other cereal products used in human nutrition, and studies made of the extent of phytic acid destruction in bread-making and in the preparation of other cereal foods for consumption. This latter problem is being studied at this Institute at the present time.

SUMMARY.

The total phosphorus and phytic acid phosphorus contents of a series of samples of wheat, bran, pollard and flour collected from four mills in Western Australia at three different times during 1947 were determined.

The variation between samples of the same material was found to be comparatively small. The average values obtained for total phosphorus, expressed as percentages on the dry basis, were as follows: wheat 0·256, bran 0·914, pollard 0·471, flour 0·090. The average values for phytate phosphorus similarly expressed were: wheat 0·172, bran 0·751, pollard 0·334, flour 0·026. The average extraction percentages, obtained from the mills, were 15, 13 and 72 p.c. for the bran, pollard and flour respectively.

The mean proportion of total phosphorus present as phytic acid phosphorus was found to be 67·1, 82·4, 70·4 and 29·4 p.c. for the wheat, bran, pollard and flour respectively.

The inorganic phosphorus content of one set of samples from each mill was obtained. The variation was small and the average results were: wheat 0·012, bran 0·039, pollard 0·025 and flour 0·004 p.c. on the dry basis.

These results were compared with overseas analyses and the conclusion reached that Western Australian wheat and mill products are substantially lower in total, phytate and inorganic phosphorus than similar products from most other parts of the world for which results were available. Data from other parts of Australia are too meagre to determine whether this finding applies generally to Australian wheat and mill products.

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THE THIAMIN AND RIBOFLAVIN CONTENTS OF WHEAT AND ITS MILL PRODUCTS

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It is now well established that neither thiamin nor riboflavin is evenly distributed throughout the wheat grain. The mill products, bran, pollard and germ, are invariably richer in these two vitamins than white flour, the actual amounts and proportions present depending upon the milling technique and the original content of the grain. The extensive literature on this subject has been well reviewed by Bailey (1944) and Holman (1946).

Comparatively little work has been published on the thiamin and riboflavin contents of Australian wheat products. Slater and Rial (1941), using the thiochrome method of assay, determined the thiamin content of a number of New South Wales wheats, of white flours from mills in all the mainland States and of a few wheat germs and breads. These workers also present data for the distribution of thiamin in mill products from one mill in New South Wales. A much more extensive series of analyses for thiamin in Australian wheat and white flour was also carried out under the direction of the Nutrition Committee of the National Health and Medical Research Council (1941 and 1942). Work on riboflavin is almost entirely confined to that of Morell (1947), who applied the fluorimetric method of Slater and Morell (1946) to the determination of the riboflavin content of wheat, flour, bran, pollard and germ from one mill in each Australian State.

MATERIALS AND METHODS.

Sampling.

Samples of wheat, bran, pollard and flour were obtained in March, 1947, from each of four mills at Cottesloe, Northam, Kellerberrin and Narrogin in Western Australia. These samples formed part of a series obtained for another purpose and the method of sampling has been previously described (Lee and Underwood, 1948). It may be stated, however, that the flour, bran and pollard samples were drawn from the mill streams so as to be as representative as possible of the wheat collected from each mill. The average extraction percentages of the four sets of samples were bran 15·25 p.c.; pollard 13·25 p.c.; and flour 71·5 p.c.

[1] Working under grants from the National Health and Medical Research Council and the Wheat Pool of W.A.

Methods of Analysis.

Thiamin. The method used was essentially that of Slater (1946). This is an improved version of the earlier method of Slater (1941) who adapted the thiochrome method of Wang and Harris (1939) for use on cereals and cereal products. Our method differed slightly from the improved method of Slater in that the method of addition of reagents was that described by Hinton (1943). Readings were made with a Spekker fluorimeter.

Riboflavin. The highly specific fluorimetric method of assay of Slater and Morell (1946) which was based on that of Najjar (1941), but differed from it in several important respects, was used. The extraction procedure recommended by Morell (1947) for wheat, flour and pollard and the special extraction procedure developed by this worker for bran was applied. Readings were made with a Spekker fluorimeter.

Duplicate determinations were carried out on the extract of each sample both with the thiamin and riboflavin assays. All the results reported are the average of duplicate determinations agreeing to within 10 p.c. If the duplicates did not at first agree to within 10 p.c. the whole assay was repeated.

TABLE 1.

Thiamin and riboflavin distribution between wheat and its mill products.

(Expressed on air-dry basis).

Milling product.	Location of mill.	Thiamin content μg./gm.	Riboflavin content μg./gm.	Products as p.c. extraction of whole wheat.	Thiamin in products as p.c. of whole wheat.	Riboflavin in products as p.c. of whole wheat.
Wheat	1. Cottesloe	3·88	0·78			
	2. Kellerberrin	3·95	0·76	100	100	100
	4. Narrogin	3·40	0·70			
	3. Northam	3·66	0·79			
	Mean	3·72	0·76	100	100	100
Flour	1.	1·54	0·27	72	28·6	24·9
	2.	1·05	0·32	73	19·4	30·8
	3.	1·42	0·26	70	29·2	26·0
	4.	1·38	0·32	71	26·8	28·8
	Mean	1·35	0·29	71·5	26·0	27·3
Bran	1.	6·7	2·22	16	27·6	45·5
	2.	8·9	2·06	14	31·6	38·0
	3.	5·2	2·46	16	24·4	56·0
	4.	5·5	2·61	15	22·3	49·5
	Mean	6·6	2·34	15·25	26·9	46·9
Pollard	1.	15·4	1·43	12	47·6	22·0
	2.	14·4	1·56	13	47·5	26·8
	3.	11·1	1·22	14	45·6	24·4
	4.	11·7	1·14	14	44·8	20·2
	Mean	13·2	1·34	13·25	47·0	23·4
			Mean total percentages	100	99·9	97·6

EXPERIMENTAL RESULTS.

The thiamin and riboflavin contents of the different wheat products, expressed on the air-dry basis, together with their distribution between the wheats and the various milling products, are presented in the accompanying Table. The mean moisture contents of the samples were as follows: wheat 12·0, bran 11·4, pollard 11·8, and flour 12·6 p.c. The percentages of thiamin and riboflavin contributed by the flour, bran and pollard should, in each case, total 100, assuming that there has been no separation of germ. In any case many workers have shown that the contribution by the germ to the total thiamin and riboflavin in wheat is only about 1–2 p.c. The figures actually obtained, by calculation from the mean extraction figures and the mean contents of the products from the four mills, were 99·9 p.c. in the case of thiamin and 97·6 p.c. for the riboflavin. This provided a very satisfactory check on the accuracy of the methods used.

The standard errors of the means are not given as the numbers of samples are small and the individual results are presented. It is apparent from the Table, however, that the variability between mills is comparatively small, particularly in the case of riboflavin. An exception is provided by Mill 2 (Kellerberrin) in the case of thiamin. This mill has produced a flour relatively poor, and a bran unusually rich, in thiamin. It is interesting to note that the riboflavin contents of the flour and bran from this mill do not differ greatly from those of the other three mills. Apparent discrepancies of this sort can probably be explained from the fact that thiamin and riboflavin are not distributed in the same way throughout the different parts of the wheat grain. Thus Andrews (1943) reported thiamin to be 5·80:1 times more concentrated in the germ half of the wheat kernel than in the brush half, whereas riboflavin was only 1·81:1 times more concentrated.

DISCUSSION.

The principal purpose of the present investigation was to study the distribution of thiamin and riboflavin among milling products under the conditions of milling usual in Western Australia. A much more extensive study of the thiamin and riboflavin contents of Western Australian wheats, and the factors influencing them, is already in progress and will form the subject of a later publication. It is of interest, however, to compare briefly the values obtained with previously published analyses.

Morell (1947) obtained the following average values for the riboflavin content of the products of six Australian mills: wheat 0·84; flour 0·27; bran 2·35 and pollard 2·07 μg./gm. For wheat, flour and bran these values are very similar to those of the present study, viz. 0·76, 0·29 and 2·34. Our average value of 1·35 μg./gm. for pollard, is much lower than Morell's, however. This confirmation of Morell's findings, at least in so far as W.A. wheat, flour and bran are concerned, may be of some nutritional importance because as this worker showed, the values obtained are appreciably lower than those obtained in most overseas investigations.

Our results for thiamin are not in quite such close conformity with other Australian data, although the mean values for bran and pollard are very similar to those given by Slater and Rial (1941) for these products from one N.S.W. mill, viz. 7·4 and 12·8 μg./gm. Three of our four samples of flour also lie within the range found by Slater and Rial. In the investigation sponsored by the Nutrition Committee of the National Health and Medical

Research Council 264 Australian wheats were found to range in thiamin content from 3·40 to 6·92 μg./gm. (mean 5·02) in the 1941 season and 45 samples from 3·5 to 6·6 μg./gm. (mean 5·17) in the 1942 season. 129 samples of white flour from 108 mills throughout Australia were also examined. The true weighted mean of these flours was calculated to lie between 1·56 and 1·79 μg./gm. but the range was very large. Nineteen of the samples contained between 1·00 and 1·39 and three between 2·60 and 3·19 μg./gm.

Our wheats and flours all fall within the above ranges but are entirely restricted to their lower parts. If this finding is substantiated for Western Australian wheat and flour generally, it points strongly to the necessity for careful study of the factors influencing the thiamin content of these materials, especially as the Australia-wide investigations just mentioned have stressed that average Australian wheat and flour is considerably richer in thiamin than these products grown and milled in England and America.

The figures for the distribution of thiamin and riboflavin among the milling products show that in each case almost three-quarters of the amount present in the wheat is lost, so far as direct human consumption is concerned, to the mill offals. The distribution between these offals is very different, however, for the two vitamins. Nearly half the total thiamin of the wheat is contained in the pollard fraction, the rest being evenly divided between the bran and the flour. The total riboflavin, on the other hand, is fairly evenly divided between the flour and the pollard, and almost half is present in the bran. In the case of thiamin, these results are not greatly dissimilar from those obtained in the other Australian investigations mentioned above. Thus Slater and Rial (1941) in their work on the products of one mill in New South Wales found the distribution of thiamin to be as follows: flour 29·6, bran 17·3 and pollard 53·1 p.c. of that originally present in the wheat. The mean percentage distribution for eight mills, obtained in the larger investigation mentioned above, was flour 26·1, bran 19·8 and pollard 54·1. These may be compared with our mean values from four Western Australian mills, of flour 26·0, bran 26·9 and pollard 47·0 p.c. The higher figure obtained by us for bran is no doubt due to the much higher extraction percentage, namely, 15·25 p.c., compared with 7·4 and 11·1 in the above investigations.

The only Australian work on riboflavin distribution with which our results may be compared is that of Morell (1947) on the products of six Australian mills. Agreement is very good in that Morell also found that about three-quarters of the total riboflavin was lost in the mill offals, but his figures for the distribution of this vitamin between the bran and pollard are very variable, due largely to very variable extraction percentages. There is no clear evidence from his data, however, that the bran contributes very much more than the pollard, as found in our investigation.

SUMMARY.

The thiamin and riboflavin contents of wheat, flour, bran and pollard from four widely separated mills in Western Australian were determined. For thiamin the thiochrome method as adapted for use on cereals by Slater (1946) was used. For riboflavin the fluorimetric method of Slater and Morell (1946) was used, together with the extraction procedures for wheat, flour, pollard and bran described by Morell (1947).

The average values for the four wheats, flours, brans and pollards were 3·72, 1·35, 6·6 and 13·2 μg./gm. for thiamin and 0·76, 0·29, 2·34 and 1·34 μg./gm. for riboflavin respectively, all expressed on the air-dry basis. The variability between mills was small for each product in the case of riboflavin, but was fairly large in the case of thiamin, except with the wheat.

The extraction percentages did not differ greatly from the four mills. They averaged 71·5, 15·25 and 13·25 p.c. for the flour, bran and pollard respectively. Using these figures it was calculated that, of the total thiamin present in the wheat, an average of 26·0 p.c. remained in the flour, 26·9 p.c. was contributed by the bran and 47·0 p.c. by the pollard. The appropriate figures for riboflavin were 27·3, 46·9 and 23·4 p.c.

These results were compared with those of other Australian investigations.

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OBSERVATIONS ON THE BIOCHEMICAL AND SEROLOGICAL CHARACTERISTICS OF *PSEUDOMONAS PYOCYANEA*

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The genus Pseudomonas includes bacilli which are gram-negative, non-sporing, aerobic, ammonia-forming and motile by means of polar flagella. They show little ability to ferment carbohydrates but frequently liquefy gelatin. They mostly produce a water-soluble pigment which may be blue, green, yellow, purple or brown in colour as well as a yellow, fluorescent, water-soluble substance. They are common in soil and water, many are plant pathogens and some are connected with pathological conditions in man. The pigments are derivatives of phenazine and Tobie (1945) has suggested that the genus Pseudomonas should comprise those bacilli which produce phenazine derivatives or water-soluble fluorescent pigments or both. The relationship, if any, between the phenazine and the fluorescent pigments is unknown.

The species, *Ps. pyocyanea*, first isolated by Gessard in 1882, is widely distributed in nature being found in water, sewage and sometimes on the normal skin, particularly of the axilla and perineum (Ruzicka, 1898). It is not infrequently found in wounds, where it gives rise to the blue pus from which it has received its name. It may invade the middle ear, meninges, bronchi and other organs and even invade the blood-stream (Williams and Cameron, 1896). According to Pons (1927), it is especially pathogenic in the tropics where it may give rise to typhoid-like infections and abscess of the liver. It is otherwise regarded as an organism of low-grade pathogenicity and is usually associated in wounds with other organisms, of which *Staphylococcus pyogenes* is common. It is pathogenic for animals if injected in sufficiently large doses and may cause death within 24–48 hours. Different strains vary in virulence.

In culture, *Ps. pyocyanea* produces a bluish-green pigment which diffuses through the medium. Two substances are connected with this pigment, one, pyocyanin, is green in colour, non-fluorescent and soluble in chloroform or water, the other, fluorescin, being yellow, fluorescent, soluble in water but insoluble in chloroform. In old cultures a brown or black pigment forms from the oxidation of the green pigment. Jordan (1899) divided his series into four varieties: (1) those producing both pigments, (2) those producing pyocyanin only, (3) those producing fluorescin only and (4) those producing neither.

Gessard (1892), working with strains producing both pigments, was able to produce varieties giving pyocyanin or fluorescin only as well as some which produced neither.

Strains isolated from water and closely related to the species *Ps. pyocyanea* but with a lower optimum temperature and less pathogenicity for animals have been regarded as of a separate species, *Ps. fluorescens*. With some strains difficulty has been found in deciding to

which species they belong and Sandiford (1937) was inclined to regard strains with the characteristics of the species *Ps. fluorescens* as members of the other species which had become adapted to a saprophytic existence. Strains isolated from water could not be distinguished from those of human origin by means of the cultural characteristics which Bergey (1934) employed to differentiate the two species.

Efforts to sub-divide strains on the basis of biochemical tests have given a large number of groups. Tanner (1918) was able to divide 42 strains into 27 groups. Seleen and Stark (1943) in a study of 199 strains, were able on the grounds of ability to liquefy gelatin, affect milk or reduce nitrates, to form 14 groups, with further sub-groups on ability to produce pyocyanin and to utilize sucrose, acetic acid, lactic acid or tartaric acid as sole source of carbon.

Reports on the serological identity or otherwise of strains of *Ps. pyocyanea* do not agree. Meader, Robinson and Leonard (1925) concluded from the results of absorption tests that the organisms were serologically uniform. Aoki (1926) on the other hand, found 22 types in an examination of 50 strains. Elrod and Braun (1942) and Munoz, Scherago and Weaver (1945) also found the group to be serologically heterologous. Gaby (1946), who made an intensive study of the variants from seven strains, found that there were three basic colony forms, that the flagellar agglutinogens of identical colony forms were alike but that they were different from those of dissimilar colony form, all forms having a common somatic antigen.

The investigation which forms the subject matter of the present report was undertaken with two practical purposes in view. The first purpose was to obtain an anti-serum or mixture of anti-sera which could be used for the rapid identification of strains of *Ps. pyocyanea*, preferably by slide-agglutination; this would be particularly helpful with strains which produce little pigment on agar or which are isolated on media such as blood agar or MacConkey's agar where the characteristic pigment is not easily recognized. The second purpose was to provide, if possible, typing sera, to attempt to trace the origin of the organisms which invade damaged tissues and to assist in deciding whether these organisms came from the patient or were the result of cross-infection. Both of these purposes have been, to a certain degree, achieved.

MATERIAL AND METHODS.

Strains Used.

One hundred and thirty-eight strains, isolated from faeces, urines, suppurative conditions, bile, sewage, water and contaminated media were examined. Ten stock strains were of unknown but probably human origin. Organisms with the biochemical characteristics of *Ps. pyocyanea* but producing no typical green or yellow pigment were not included. Strains were numbered serially as collected, P1, P2, P3, etc.

Preparation of Anti-sera.

The organisms were incubated for 18 hours on nutrient agar slopes, the temperature of incubation being 37° C. for the strains of human origin and 25° C. for those which failed to grow at 37° C. The organisms were then suspended in normal saline containing 0·2 p.c. formalin, to give a concentration of approximately 8,000 million per ml., incubated for 4 hours at 37° C. and stored at 4° C. Rabbits were inoculated intravenously twice weekly for

three weeks, the volumes of the successive inocula being 0·1, 0·2, 0·4, 0·7, 1·0 and 1·0 ml. Four days after the last injection, blood was withdrawn by heart puncture. The serum was separated, preserved by the addition of chloroform and stored at 4° C.

Preparation of Suspensions for Agglutination.

Suspensions of living organisms were prepared by washing off the organisms from an overnight agar slope culture with 1·5 ml. of saline, the resulting concentration being approximately 20,000 million per ml. Suspensions for tests for somatic antigens were prepared from these by suspending the organisms, separated by centrifugalization, in 1·5 ml. of absolute alcohol, maintaining them at 45° C. for 30 minutes and resuspending them, after further separation by centrifugalization, in the original volume of saline.

Technique of Slide Agglutination.

With the aid of a Pasteur pipette, a drop of suspension was placed on a slide and mixed with an equal-sized drop of diluted serum. The slide was then rocked for two minutes and the result read with a hand lens.

Haemolysis.

This was detected on nutrient agar plates to which 5 p.c. sheep blood had been added. Horse blood was found to be less suitable than sheep blood since incubation for several days was necessary for haemolysis to show by which time the horse red-cells had begun to lyse throughout the whole of the medium. The various strains were "spot-inoculated" on the medium, six strains being applied to each plate. Incubation was carried out at 25° C., since some strains failed to grow at 37° C. and it was desired to carry out the tests at the same temperature for all strains for comparative purposes.

Absorption of Anti-sera.

The organisms to be used for absorption were grown on agar slopes for 18 hours, washed off with saline and separated by centrifugalization. The serum was diluted 1:5 with saline and 0·5 ml. added to the organisms from 1, 2, and 4 slopes in separate tubes. After being allowed to stand for one hour at 37° C., the organisms were removed by centrifugalization.

RESULTS.

Biochemical Tests.

All strains from human sources grew well at 37° C. and at 25° C. Of the twelve strains from non-human sources, e.g. sewage, water and contaminated media, all grew well at 25° C. but very poorly or not at all at 37° C. with two exceptions. These exceptional strains were isolated from water samples.

Most of the strains were haemolytic. Two kinds of haemolysis were noticed, one resembling the β haemolysis and the other the α prime haemolysis of the streptococci. In most instances, the haemolysis appeared in two or three days. Further incubation produced extension of the zones, some of the strains which at first showed only partial haemolysis, developing inner zones of complete haemolysis while others showed merely extension of the zones of incomplete lysis. Ten strains showed no haemolysis after seven days. These were the ten strains of non-human origin which failed to grow well at 37° C.

Attempts were made with one of the markedly haemolytic strains to produce a cell-free filtrate containing haemolysin. The organisms were grown at 37° C. and 25° C. in nutrient

broth with and without 0·1 p.c. agar and with and without increased CO_2 pressure. Incubation for 1, 3 and 7 days was allowed. The liquids were then clarified by centrifugalization and filtration through a Seitz EK filter and the filtrates tested against washed sheep red-cells at 37° C. No haemolysis was detected.

Pigment, blue-green or greenish-yellow, formed on all agar slope cultures, to a degree which varied from strain to strain. After a few days at room temperature, the colour changed to yellow and then to brown. Two strains formed a brownish-black pigment, apparent within 24 hours. In the deeper parts of the medium on which these two strains had grown, blue-green pigment was visible below the darker surface layer, suggesting that the pigment system in these two strains was similar to that in the others but that the oxidation process by which the brown pigment is produced from the blue one was unusually rapid.

The presence of fluorescin was detected by holding tubes containing broth cultures, in the rays of a mercury lamp and viewing the tubes against a dark background. Some strains gave a strong fluorescence within 24 hours, some showed very little after a week's incubation. Varying degrees of reaction between these extremes were found. There was no fading of the reaction once it appeared, as with the blue pigment. Formation of pyocyanin did not parallel that of fluorescin.

All strains, with one exception, were actively motile when first isolated. After 6 months, one strain originally motile was found to be non-motile and no longer agglutinable with a flagellar anti-serum (see below) although it had been originally agglutinable. A sub-culture of the stock strain which had been preserved under paraffin was found to be still motile and agglutinable. The motile form possessed a single well-formed flagellum. No flagella could be demonstrated on the other form. Both forms of the organisms produced pyocyanin to the same degree and showed no differences in their other biochemical properties.

A second strain was non-motile, even on primary isolation. It gave very mucoid colonies, resembling those of Friedländer's bacillus. After several sub-cultures, the mucoid nature of the growth became much less marked. A third strain, on the primary isolation plate, showed two colony forms, one motile and the other non-motile. That the two were related and not merely two forms accidentally occurring together, was supported by the fact that both produced dark-brown pigment within 24 hours on nutrient agar, an appearance shown by only one of the other strains in the collection.

In litmus milk, most strains produced some clotting with subsequent solution, with little differences other than in degree and in rate of reaction. Three strains produced peptonization without previous clotting as shown by a gradual clearing of the medium.

Lactose was not fermented by any strain.

Since *Ps. pyocyanea* is an active producer of ammonia resulting in an alkaline reaction to broth in which it has grown, it was possible that lactose fermentation, if it occurred, would be masked by the simultaneous production of alkali, since the method used to detect fermentation was the noting of colour-change in an acid-base indicator in the medium. To examine this possibility, tubes of peptone water containing 1 p.c. lactose were inoculated with a strain of the organisms and incubated for varying times. Seitz filtrates of the cultures were then examined for reducing sugar by the Hagedorn and Jensen technique as used for estimating sugar in blood. The titrations showed that, taking the titre for the uninoculated controls to represent 1·00 p.c. the figures for 1, 3 and 7 day cultures were 0·903, 0·927 and 0·988 p.c. respectively. To reduce ammonia-formation and complications due to the presence of peptone, lactose broth was prepared using Koser's citrate medium instead of peptone water as base. Again taking the titration figure for the controls to be 1·00 p.c., the corresponding figures for two 7-day cultures were 1·00 and 1·05 p.c. A parallel set of tubes of this medium with indicator added showed no colour change indicative of acid formation. As a result of these tests, it was concluded that lactose was not fermented.

No strains produced indole within 5 days when tested by the method of Holman and Gonzales (1923). Atypical positive reactions were obtained when Ehrlich's indole reagent was used; the reactions showed more in the medium than in the layer of reagent on top. This has been attributed by Sandiford to the action of the acid in the reagent on the pigment of the organism and not to indole.

All strains formed catalase and were able to utilize citrate as their sole source of carbon. All produced ammonia in peptone water.

On potato slopes, some strains produced much blue-green pigment and some very little. After incubation for a week at room-temperature, a brownish, slimy growth had formed from all strains with varying amounts of blue pigment still evident.

Fibrinolytic activity, as shown by clearing of heat-precipitated fibrinogen on fibrinogen agar plates (Christie and Wilson, 1941), was shown by all strains. Some produced zones of faint opacity as well. No such zones were produced on serum agar. The lysis was not very marked or distinct as it is with staphylococci but resembled more the weak reactions given by fibrinolytic streptococci. Attempts were made to obtain cell-free culture filtrates containing fibrinolysin as has been done with staphylococci (Christie, Graydon and Woods, 1945) but only very weak reactions were obtained.

No lipolytic activity was shown when strains were grown on butter-fat agar plates.

Dextrose was fermented by 59 strains in 1 day, by 27 strains in 2 days, by 15 strains in 3 days, by 12 strains in 4 days and by 1 strain in 6 days. No fermentation was given by 24 strains in 14 days. The tests were repeated with the non-fermenters and the slow fermenters using Koser's citrate medium as base instead of peptone water. Similar results were obtained except that fermentation was less rapid.

Fifteen strains of human origin and six strains of non-human origin were inoculated from 24-hour agar slope cultures into oxalated human plasma diluted 1:5 with saline. The tubes were incubated at 37° C. and 22° C. for 24 hours. No coagulation was detected.

Gelatin stabs were inoculated from 85 strains and incubated at 37° C. and 22° C. Tests for liquefaction were carried out by placing the tubes at 4° C. for one hour and then inverting. Some strains caused rapid liquefaction, others required incubation for periods varying from two to fourteen days, while twelve strains failed to cause liquefaction within fourteen days. The percentage of non-liquefiers was higher amongst the strains of non-human origin than amongst the others. The tests, repeated with two other batches of medium, gave results which were approximately but not strictly parallel.

Flagellar staining showed that most strains possessed one flagellum but the results were not always straightforward. Preparations of strains showing predominantly one large well-formed flagellum not infrequently showed occasional bacilli with several apparent flagella bunched at one pole. These, however, were variable in size and number with as many as 14 per bacillus; they were finer, shorter and less regular than the single flagellum shown on other bacilli in the same preparation. An occasional bacillus showed a definite envelope, unstained but with a stained outline and with the flagellum attached to the outside of this envelope and showing no sign of being attached directly to the inner cell. The appearances agreed with Pijper's (1946) suggestion that with flagellated bacilli there is a surrounding capsule-like material of which the flagellum is an extrusion and that on drying artefacts with the appearance of flagella can originate from disruption of this substance. Where more than one flagellum appeared, it was usually one of two or more attached to one pole and in such instances was smaller and finer than the single flagellum shown on neighbouring bacilli. It was concluded that normally strains possess one flagellum only.

No flagella were seen in stained preparations of the three non-motile strains in the collection.

Serological Tests.

Thirty-five antisera were prepared, each being given the number of the strain from which it was prepared. No animal failed to give a good response except in cases where there was reason to suspect that degenerate strains were used. The average end-point in titration of sera by slide-agglutination was about 1:160 and in titration by the tube method, in a trial series, about ten times greater. All results given below were obtained by slide-agglutination.

Serum 1 agglutinated eight of the first eleven strains to an appreciable but not identical titre, two strains (P3 and P4) to a low titre and strain P2 not at all. The lowest dilution used was 1:10. When the serum was absorbed with a suitable dose of organisms of strain P4, its agglutinins for strains P3 and P4 were simultaneously removed. These tests were carried out using suspensions of living organisms in saline. When carried out with alcoholized organisms, the agglutination of strains P3 and P4, using unabsorbed serum, did not occur, suggesting that this had been due to common flagellar antigen.

Serum 3 agglutinated all eleven strains except strain P2 but gave a much higher titre against organisms of strain P3 than against any of the others, when living organisms were used. When tested against organisms in the O phase, the serum was specific for strain P3.

Similar results were obtained with serum 4 and strain P4.

Serum 2 was highly specific. It agglutinated strain P2 to a titre of 1:160 with the organisms in the O or O-H phase but none of the other eleven strains in either phase.

Strain P15 reacted less strongly with serum 3 than did strain P3. Serum 15 reacted in the reverse manner so that by using each serum suitably diluted (1:40) they were made to agglutinate the homologous strain only. It was at first thought that the two strains had each two antigens, one specific and one common but as more strains related to these two were encountered, it was found that they did not separate into two clear-cut groups but that gradation in quantity of either antigen occurred. These strains have, therefore, been classed as one group.

Serum 13 agglutinated strain P13 but not strain P1. Serum 1, on the other hand, agglutinated both strains. A month later serum 1 also failed to agglutinate strain P1. When strain P1 was sub-cultured from the culture of the original strain preserved under paraffin, it reacted equally well with both sera. This was evidently another instance of the sharing of antigens, strain P1 having degenerated, losing first one and then the other antigen, while the antisera varied in their content of the two agglutinins. Further evidence to support this explanation on the grounds of degeneration or variation was found when colony differences and differences in pathogenicity were examined, as described below.

Strain P16 was agglutinated weakly by serum 1 when in the living phase but by none of the sera when in the O phase. An antiserum prepared from it agglutinated many strains strongly when these were in the living phase but none at all when they were in the O phase. A second serum prepared from it, using larger inocula, reacted similarly. Such O antibodies as were detected in this second serum suggested that it had an antibody similar to one of those in serum 1. It was found possible to absorb serum 1 with organisms P16 and to remove the agglutinins for strain P16 while leaving a considerable titre of agglutinins for strain P1. On account of the high titre of flagellar antibodies in serum 16 and its almost negligible somatic antibody content, this serum was used in high dilution (1:80) to test for common flagellar agglutinogens in the other strains, using suspensions of untreated organisms. Sixty-eight strains were agglutinated.

Strain P17, which was agglutinated weakly by serum 15 but by none of the others yielded an antiserum low in somatic antibodies but high in flagellar antibodies. When tested in high dilution (1:160) against living suspensions of all the strains, 37 were agglutinated. None of these 37 had reacted with the other flagellar serum.

Serum 13 had agglutinated strain 2 in the living condition to an appreciable titre but not at all in the O phase, suggesting that P13 and P2 had a common flagellar antigen distinct from the other two. It was found possible to absorb out the agglutinins for P2 while leaving the serum still capable of agglutinating the O form of strain P13. Absorption of the serum with a suitable organism with the same O but a different H antigen did not give a residue sufficiently rich in H antibody to allow this to be used as a flagellar antiserum. However, it was possible to show that many strains did not have the same H antigen as P13 since these strains were not agglutinated in the living condition by the unabsorbed serum in low dilution.

Strain P8 was agglutinated by serum 1 to a titre appreciably less than that obtained with strain P1. A serum made from strain P8, on the other hand, agglutinated both strains almost equally strongly. Other strains which agglutinated weakly with serum 1, reacted strongly with serum 8. It seemed again as if there were specific and common antigens. This was borne out by precipitin reactions. Preliminary tests on precipitin reactions with one strain (P13) had shown that a substance could be extracted from the organisms either by the formamide or the HCl method which gave a precipitin ring with anti-sera. It was later found that the supernatant fluid from centrifugalized 48 hours' broth cultures gave the same reaction. When the P1, P8 strains were tested by the precipitin method against sera 1 and 8, they could be divided into two clear-cut groups, the specific substance being apparently of a polysaccharide nature while the common substances were probably protein.

It seemed possible that the P3, P15 series might be separated in a similar manner but with these strains, there was cross-agglutination by the precipitin as well as by the agglutination technique.

Two strains P36 and P50 gave specific antisera which agglutinated the parent strains and some others, none of these strains having been agglutinated by any of the sera previously prepared.

TABLE 1.

Results of somatic agglutination tests, showing number of strains in each group.

Origin of strains.	Human.								Non-human.						
No. of anti-serum	8	1	15	2	4	50	36	×	23	24	25	46	51	60	×
No. of strains	41	21	20	12	7	6	2	17	2	1	2	2	1	1	1

Strains in Column × were not agglutinated by any of the anti-sera.

Two strains isolated from water but showing the characteristics of the human group are not entered in the table. They were agglutinated by anti-sera Nos. 8 and 1 respectively.

By means of all these sera, it was possible to type the two water-borne strains which grew well at 37° C. and all of the human strains except 17. Sera prepared from eleven of these showed no specific antibodies and it is possible that these strains were degenerate forms such as the one mentioned above in connection with strain P1.

The ten strains of non-human origin, which grew poorly or not at all at 37° C., were not agglutinated by any of the sera so far prepared. By making anti-sera from these, it was found that there were six groups. No cross-agglutination occurred with these sera, using suspensions of organisms in the O phase and very little in the living phase. Precipitin reactions were also specific. One strain, however, P46, gave a serum which agglutinated twelve of the human strains, ten of them fairly strongly, although P46 itself gave no agglutination or precipitin reaction with any of the sera prepared from human strains. The

TABLE 2.

Results of mouse pathogenicity tests with strains of Ps. pyocyanea.

	Human strains.													Non-human strains.								
Serial No. of strain	2	3	4	8	13	15	16	17	36	50	1	1A	76	22	23	24	25	45	46	47	51	60
Dose in millions of organisms																						
2,000	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_2$	$+_1$	—	$+_1$	—	$+_2$	$+_1$
1,000	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	—	—	—	—	—	—	—	$+_1$
500	$+_1$	$+_1$	$+_1$	$+_1$	$+_2$	$+_1$	$+_1$	$+_1$	$+_2$	$+_1$	$+_1$	$+_1$	$+_1$	$+_2$	—	—	—	—	—	—	—	$+_2$
250	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_2$	—	$+_1$	$+_2$	—	$+_2$	—	—	—	—	—	—	—	—
125	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	—	—	—	$+_1$	—	—	—	—	—	—	—	—	—	—	—
62·5	—	—	—	—	—	$+_1$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

$+_1$ means death within 24 hours. $+_2$ means death within 48 hours. — means survival.

reacting human strains, however, gave very weak precipitin reactions with serum 46, whereas strain P46 gave a very strong one. It was found possible to remove the common agglutinins by absorption and it is the results found after absorption that are entered in Table 1.

Another example of a connection between the non-human and the human strains was found with a serum from the human strain P19. A serum prepared from a non-motile variant of this strain was found to be relatively poor in antibody content. A second serum prepared with larger inocula including living organisms reacted with strain P19 in low dilutions only. This serum agglutinated many of the human strains as well as the non-human strain, P25, in a manner which did not parallel any of the specific reactions already described. It did not agglutinate any of the strains agglutinated by serum 46, and gave no precipitin reactions even with the parent strain.

Pathogenicity of Strains.

The pathogenicity of strains was tested by inoculating mice, weighing 30 gm., by the intra-peritoneal route, the volume of the inoculum being 1 ml. Organisms were grown for eighteen hours on nutrient agar slopes, at 37° C. for the human strains and at 25° C. for the others. They were then washed off with saline and the concentrations estimated with Burroughs Wellcome standard opacity tubes. Serial doubling dilutions were prepared commencing with suspensions containing 2,000 million organisms per ml.

Where death occurred, this happened in one or two days. The survivors were still alive and apparently normal after fourteen days.

The results are shown in Table 2. It will be seen that the lethal dose for the human strains was much smaller than that for most of the non-human strains but that there were strains intermediate in killing power in both groups.

Strain P1 showed the normal killing power for a human strain but strain P1A, an inagglutinable variant of this strain, had less pathogenicity. Strain P76, a non-mucoid variant of a strain originally giving very mucoid colonies, also shows lowered pathogenicity.

The greater lethal power of the strains of human origin may have been partly or entirely due to their ability to grow at 37° C. To test this, suspensions of strain P2, a human strain (see Table 2), and of P24, a non-human strain, were killed by exposure to a temperature of 55° C. for one hour and mice were inoculated with graded doses. The lethal dose for strain P2 was 2,000 million organisms and for strain P24 4,000 million. The difference in lethal power between the two organisms had thus been greatly reduced, suggesting that the difference in optimum temperature for growth had played a large part in deciding their pathogenicity when in the living condition.

No toxin was detected in broth cultures. Strain P2 was grown in nutrient broth for one and four days at 37° C., the broth rendered clear by centrifugalization and the mice inoculated, intraperitoneally with 1 ml. quantities and intravenously with 0·1 ml. quantities. None showed ill effects.

Variability of Strains.

Strain P1, after repeated sub-culture, became so altered that it was no longer agglutinated with serum 1. That the serum had not altered was shown by the fact that it still agglutinated other strains and that it still agglutinated strain P1 when this was revived from the original culture preserved under paraffin. Both forms of P1 produced abundance of pyocyanin and both were motile but the inagglutinable form gave a less homogeneous suspension in saline than the other and the two forms gave different colony forms when plated out on nutrient agar. After three days' incubation the colonies of the inagglutinable form were

smooth and uniform whereas the others had an irregular edge and a somewhat pitted surface. Attempts to produce agglutinable organisms from the others by treble passage through mice were unsuccessful. The degeneration had taken place in at least two stages, the first when the organisms would agglutinate with serum 1 but not with serum 13, and the second when they would agglutinate with neither although they originally agglutinated with both. The inagglutinable form was less lethal for mice than the other (see below).

The loss of motility shown by strain P19 is another form of variation that can occur *in vitro*. That this can also occur *in vivo* was suggested by the fact that when strain P53 was isolated, a motile and a non-motile form were obtained from the primary isolation plate. That they were related to each other was extremely probable since both produced, within 24 hours, dark-brown pigment, a reaction shown only by one other strain in the series.

The degeneration shown by strain P1 was shown by some other strains, originally normal, which later gave suspensions showing partial or complete auto-agglutinability and inagglutinability with antisera. Sub-cultures of these from paraffin-covered stock cultures behaved normally. A fairly large number of strains (18) was not agglutinated by any of the sera. Some of these strains could not be tested on isolation as they were received from other laboratories but this could not be said for all strains. The untyped strains gave mostly non-homogeneous suspensions in saline and were probably degenerate forms comparable with strain P1A. On two occasions agglutinable and inagglutinable forms were found together on the primary isolation plate, suggesting that the degeneration can occur *in vivo* as well as *in vitro*. Antisera prepared from eleven of these unagglutinated strains were unsatisfactory as compared with those from normal strains, in that they were all of low titre and showed little or no specificity.

DISCUSSION.

With the exception of haemolysis and optimum temperature for growth, none of the usual biochemical tests was helpful in grouping strains. Tests for fermentation of dextrose, liquefaction of gelatin and action on milk showed differences between strains, but these tended to be in degree rather than in kind and in no way agreed with serological type or source. It is questionable if these differences have any significance. Liquefaction of gelatin has been given some significance in the past, but the results obtained in this survey indicate that its value as a differential test is very limited, since the results vary slightly with the batch of medium used, there is a broad range between the rapid liquefiers and the non-liquefiers so that a lengthy time of incubation must be allowed and, finally, the division obtained means little since it correlates with no other characteristic.

Haemolysis, on the other hand, correlates with optimum temperature for growth and with source. All non-haemolytic strains were of non-human origin and unable to grow well at 37° C. The test for haemolysis is a clear-cut one. All strains from pathological conditions as well as all strains which grew well at 37° C. were haemolytic. Salvin and Lewis (1946) found that all strains of *Ps. pyocyanea* (*aeruginosa*) were haemolytic on ox blood agar, while strains of *Ps. fluorescens* were non-haemolytic.

The lesser pathogenicity of the non-human strains as compared with the others is of reduced significance when it is borne in mind that it may be largely the result of their lower optimum temperature for growth. The tests lose further value on account of the fact that, like fermentation of dextrose and liquefaction of gelatin, they give intermediate results with strains of both groups.

The non-human strains tested in this survey are apparently similar to those referred to by other workers as of the species *Ps. fluorescens*, but, as is generally stated, the distinguishing tests for this species are at present inadequate. The members may be regarded as saprophytic forms of *Ps. pyocyanea*, but if they are considered to have properties as a group, sufficiently distinct from those of the group found in lesions, to warrant being placed in a separate species, the results found above suggest that the distinguishing tests be inability to haemolyze sheep red cells and to grow well at 37° C.

The ability to produce pyocyanin can vary from strain to strain as can the ability to produce fluorescin, so that the apparent inability to produce either may be merely an indication that the test to which the strain is submitted is not sufficiently sensitive to detect weak reactors, or that the strains found negative in either test differ from normal strains in a manner which will cause them to be regarded merely as different varieties and not necessarily as members of a different species or sub-species.

The Society of American Bacteriologists uses the species name *aeruginosa* on priority grounds instead of *pyocyanea* as used by English writers. Since it is evident that there is a pathogenic group which is capable of causing the formation of "blue pus" and a saprophytic group incapable of doing so, it might be a rational procedure to use the name *pyocyanea* for the former and the name *aeruginosa* for the latter, the use of the name *fluorescens* being discontinued.

Further differentiation of strains is possible, as has been shown, by serological tests, using slide-agglutination with the organisms in the O phase or the precipitin test with the supernatant fluid from broth cultures. In preparing antisera, excessive dosage of the animals should be avoided, since this is liable to produce antibodies which detect nonspecific antigens.

Apart from the divisions thus obtained, further subdivisions can be made according to which flagellar antigen the organism possesses. At least two common flagellar antigens are detectable, a reacting strain possessing either independently of its somatic antigen. There is some indication of a third less common flagellar antigen possessed by a few of the strains showing neither of the two common ones, while a fourth is postulated to accommodate those strains possessing none of these three. No antiserum has, as yet, shown any antibodies capable of detecting H antigens with water-borne strains.

The number of groups shown in Table 1 is much smaller than the number (22) found by Aoki, but if it is remembered that each group shown can be

divided into two or more types on the basis of flagellar antigen, closer agreement is apparent. Gaby's finding, that there is a common somatic antigen and one of three flagellar antigens possessed by each strain, is explainable on the assumption that, since he worked with a small number of strains (7), they all belonged to the commonest group shown in Table 1.

The number of groups found amongst the water-borne strains is unexpectedly high considering the small number of strains examined, and the probability is that there are still more to be found. If the six groups found were the only ones, it is most unlikely that they would all have been found in the first ten strains tested.

An effort was made to detect the source of strains which invade wounds. Swabs were taken from the ear, nose, throat, axilla, perineum and rectum of 12 patients with no history of infection. Examination failed to reveal any strains of *Ps. pyocyanea*, as did also an examination of 12 samples of normal faeces. A series of cases occurred, however, where it was possible to show that three patients with wounds had become infected from a fourth in the same ward. All four strains had the same flagellar antigen and the same somatic antigen (antiserum 4). Analysis of the distribution and numbers of strains in each group showed that the probability of four strains of this kind occurring by chance in the same ward was negligible ($P=0{\cdot}0003$).

The presence of *Ps. pyocyanea* in water samples is of some significance to Public Health Officials, especially in tropical areas. Strains found in routine analysis, however, can be disregarded if they are non-haemolytic on sheep blood agar.

SUMMARY.

An examination of 138 strains of *Ps. pyocyanea* has shown that they can be divided into a pathogenic and a saprophytic group.

The differentiating tests are ability or otherwise to haemolyze sheep red cells and to grow well at 37° C.

It is suggested that the species name *aeruginosa* be used for the saprophytic group.

The organisms may be further sub-divided into groups by slide-agglutination on the basis of somatic antigen. There are at least 7 groups in the pathogenic and 6 in the saprophytic group. There is also evidence that the strains may further differ in their flagellar antigens.

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OBSERVATIONS ON THE DEVELOPMENT OF HUMAN MALARIAL PARASITES IN THE MOSQUITO

I. MORPHOLOGICAL CHANGES

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The general developmental cycle of malarial parasites has been well known for many years, and fairly detailed accounts have been published (e.g. Wenyon, 1926). Nevertheless, the sequence of events in the early stages has not been clearly defined, and, apart from Heydon's (1923) general observations, and Roberts' (1943) comparative studies of *Anopheles punctulatus farauti* and *A. annulipes*, nothing was known of the behaviour of Melanesian strains in the mosquito, or of their temperature-growth-rate relations.

From 1943 to 1946, while we were members of the entomological team of the L.H.Q. Medical Research Unit (A.I.F.), Cairns, North Queensland, we had the opportunity of observing the mosquito cycle of malarial parasites in considerable detail. In the present paper we record some observations on the morphology of various stages, and their growth in relation to season. In a second part, we will discuss the factors which influence the establishment of infection in the mosquito.

The strains of malarial parasites were derived from soldiers infected in New Guinea, and the mosquitoes used belonged to the important vector species of the region *Anopheles punctulatus punctulatus* Dönitz.

METHODS.

Examination of Parasites in the Mid-gut of the Mosquito.

In order to observe the early stages in the gut of the insect host, mosquitoes were placed singly in 3 × 1 inch glass tubes covered with fine mosquito netting. Each mosquito was allowed to bite the gametocyte carrier through the netting, and to engorge fully—a process which usually took two or three minutes. The mosquitoes were then killed and dissected at suitable intervals after completion of feeding. Later stages were observed in mosquitoes taken from batches of 200 or more fed on the donor at the same time.

Preparations of the gut contents were made in two ways. In one, the distended mid-gut was withdrawn, together with the other abdominal organs, by nicking the inter-segmental membranes between the sixth and seventh segments of the abdomen and gently pulling out the organs into a drop of saline (0·9 p.c. NaCl). The mid-gut was then dissected away from the rest of the alimentary tract, and its rather viscous contents were mixed with just sufficient saline to permit easy spreading.

In the second method, the abdomen was cut away from the thorax with a triangular needle. The distended mid-gut was not injured by this manoeuvre, and, by holding the tip of the abdomen by the point of one needle and gently rolling a round needle over the abdomen from tip to base, the contents of the mid-gut could be squeezed out free from other abdominal organs.

To enable a comparison of the density of parasites in different mosquitoes to be made, a series of circles of known diameter from 5 mm. to 20 mm. were drawn on white paper, which was then pasted on a convenient block of wood. The slide with the dissected mid-gut was placed over the circles, and one chosen which was judged suitable for the amount of material to be examined. Before digestion had proceeded very far, large circles (17 or 20 mm.) were necessary, but after 24 hours digestion, the contents could be accommodated on smaller areas, e.g. 12 or 15 mm. diameter. The gut contents were spread over the chosen area as quickly and evenly as possible and the film allowed to dry.

It was important not to have too much saline, if imperfect spreading and distortion of the parasites were to be avoided. Gut smears were dried, and then stained with Leishman's stain exactly as for a blood film. The diameter of the microscope field was determined and a factor calculated for each circle in use. It was then easy to calculate the number of parasites per gut.

We had little success with thick films of gut contents, although on some occasions they proved useful. Considerable distortion of the parasite occurred, and the interpretation of the film was much more difficult than with thinner films.

Protozoan organisms other than malaria parasites were sometimes found in gut smears. Two of these may be mentioned here, as they might possibly be confused with *Plasmodium*. One was a small flagellate, kindly identified for us by Dr. C. M. Wenyon as *Pleuromonas jaculans*. This organism sometimes contracted into a round, darkly-staining mass, which with its two red-staining flagella could be mistaken for an abortive exflagellation in early gut smears. The other, which was not identified, was apparently the same as the "fish-like bodies" seen by Gibbins in *Anopheles* mosquitoes in Uganda (Gibbins, 1932). In late gut smears it might be confused with a vermicule. It was, however, longer and broader and was unpigmented. It did not stain so intensely, and lacked the characteristic shape of the latter. Neither of these organisms caused any confusion when they occurred in thin, well-stained portions of the film, but were sometimes hard to identify when overstained or partially obscured by other cells.

Later Examinations.

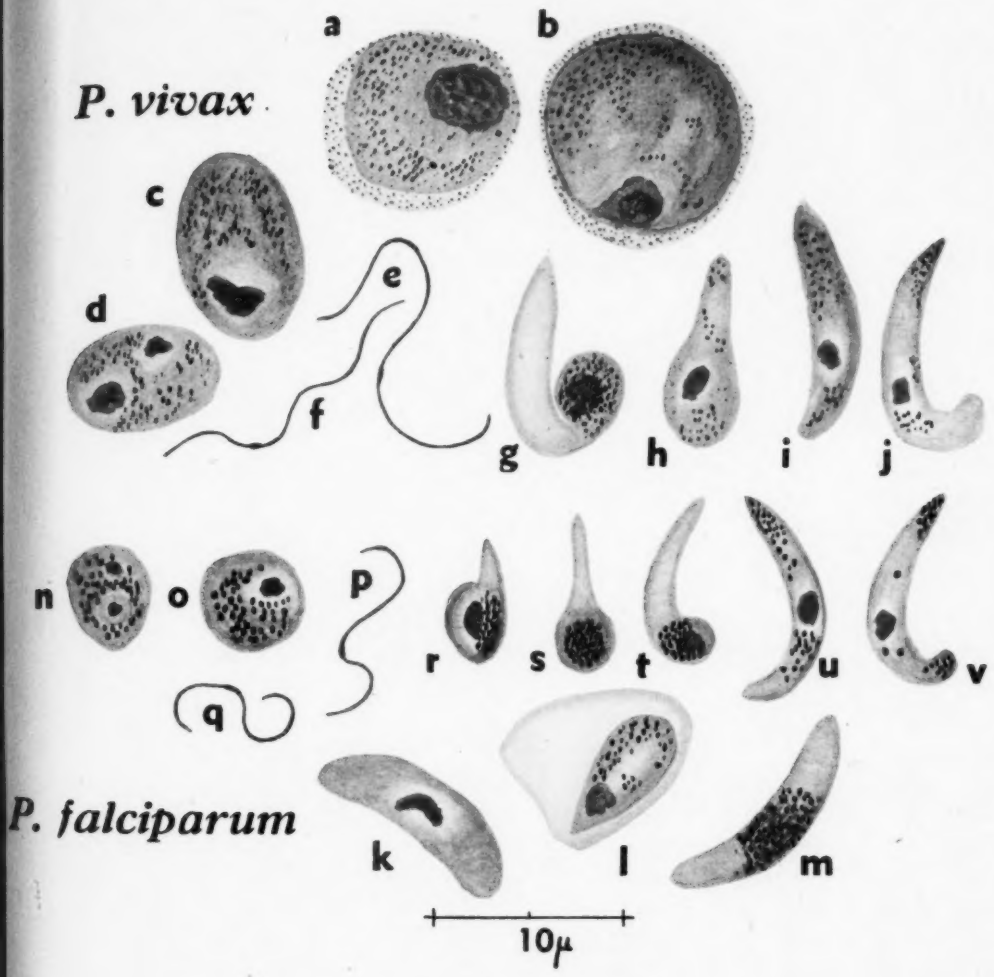
Dissections were made in 0·9 p.c. NaCl., and preparations of the mid-gut and salivary glands were examined unstained in this fluid. Permanent preparations were made of infected material, which was fixed and stained by a number of standard methods. The most satisfactory mid-gut preparations were those fixed in formal-acetic-alcohol, or in pure methyl alcohol. and stained with Delafield's haematoxylin. Sporozoites were fixed with methyl alcohol and stained with dilute Giemsa or iron haematoxylin.

THE SPOROGENOUS CYCLE.

Formation of Zygote.

Mature male and female gametocytes of both species became extracellular almost immediately after entering the gut, appearing as refractile spheres, with the pigment arranged near the centre and exhibiting an energetic dancing motion. No sign of even incipient microgamete formation was ever seen in quickly dried, thin blood films, but exflagellation

P.



EXPLANATION OF THE PLATE.

a–j. *P. vivax*; **a** male, and **b** female gametocytes from the peripheral blood; **c** and **d** macrogametes in 10-minute gut smear; **e** and **f** microgametes from the same smear; **g** developing vermicule, retort-shaped stage, from 12-hour gut smear; **h** developing vermicule from 10-hour gut smear; **i** and **j** fully-developed vermicules from 24-hour gut smear.

k–v. *P. falciparum*; **k** non-pigmented female gametocyte from patient treated with atebrin; **l** young gametocyte; **m** male gametocyte; **k, l** and **m** from the peripheral blood; **n** and **o** macrogametes from 30-minute gut smear; **p** and **q** microgametes from 10-minute gut smear; **r** developing vermicule from 12-hour gut smear; **s** developing vermicule (nucleus obscured by pigment) from 21-hour gut smear; **t** developing vermicule, retort-shaped stage from 21-hour gut smear; **u** and **v** fully-developed vermicules from 24- and 26-hour gut smears.

occurred very rapidly in the mosquito, or in wet preparations *in vitro*, fully-formed microgametes being commonly found in gut-smears made five minutes after the mosquito ceased feeding at room temperatures about 77° F. (c. 25° C.). It was sometimes delayed longer than five minutes, depending on temperatures, particularly in *P. falciparum*, but we did not see a male cell begin exflagellating later than thirty minutes after ingestion. They sometimes remained active for about forty minutes, but they could not be found in films made later than this; apparently those which fail to fertilize a female cell soon disintegrate.

The fully developed, free microgametes measured 20–30μ in length in *P. vivax*, and 12–22μ in *P. falciparum*, the thickening in each, usually regarded as the nucleus, being situated about one-third of the length from one end (Plate 1: e, f, p and q). A considerable mass of cytoplasm, pigment and possibly chromatin was left after exflagellation, the whole of this residual body staining very deeply.

Fertilization was observed as early as ten minutes after ingestion (25° C.), and was seen at various times up to forty minutes after feeding, but we formed the opinion that macrogametes, which failed to become fertilized within this period, rapidly disappeared.

The most striking features of an actual fertilization were the extremely rapid disappearance of any portion of the microgamete which did not pass into the female cell, and the intense agitation of the contents of the female cell while the act of fertilization was occurring; the whole of the cell seemed to be boiling up.

In stained preparations of mid-gut contents many female gametocytes showed two chromatin masses. We certainly noticed more binucleate forms at this stage than in the peripheral blood. Quite frequently one of the chromatin bodies was smaller than the other and very close to the surface of the organism, as if it were about to be extruded. The appearance of these bodies suggested that a division of the nucleus had occurred as a preliminary to fertilization (Plate 1: d and n).

However, many parasitic Protozoa remain in the haploid state for the greater part of their life cycle. This is true of the following organisms, in which the reduction occurs at the first division of the zygote:

Diplocystis schneideri, 6 chromosomes to 3 (Jameson, 1920).
Gregarina blattarum, 6 chromosomes to 3 (Sprague, 1941).
Aggregata eberthi, 12 chromosomes to 6 (Dobell and Jameson, 1915; Dobell, 1925).
Adelina deronis. 20 chromosomes to 10 (Hauschka, 1943).

If *Plasmodium* conformed to this pattern there would be no reducing division prior to fertilization. Another possibility is that some of the binucleate forms were zygotes in which fusion of the male and female nuclei had not yet taken place—a process which is said to take some time.

When mosquitoes were fed on gametocyte carriers with paludrine in their blood, fertilization and ookinete formation were often normal, but the young cysts failed to grow. Evidence from trophozoite studies indicates that this drug acts at the stage of nuclear division (Black, 1946; Mackerras and Ercole, 1947). If this is general, it would suggest that there is no division of nuclei between the initial formation of the gametocytes and the initial division of the zygote nucleus in the young cyst. There would be no need for a division of the macrogamete nucleus if the cell were haploid, but where do the nuclei of the several microgametes come from?

The extremely rapid appearance of fully-formed microgametes suggests that the preliminary division of the male nucleus into five or six portions must have already taken place before the blood was withdrawn, and the failure of paludrine to affect this cell raises the speculation that these nuclei may be formed perhaps at an initial stage in the development of

the young merozoite, which is destined to become a microgametocyte. None of our preparations showed sufficient cytological detail to enable us to test this hypothesis, but the diffuse nature of the male nucleus is not inconsistent with the suggestion.

Development of Vermicule.

When fixed and stained, the recently fertilized macrogamete, or zygote, was ovoid or rounded, measuring 6–9μ in diameter in *P.vivax*, and 5–7μ in *P. falciparum*. Within the next few hours it became more compact, usually measuring 1–2μ less in diameter. It remained more or less spherical until about 10 or 12 hours after feeding, when a series of changes of shape took place. Development of the sphere into the elongated vermicule was observed to take place in two ways. Most commonly a cytoplasmic projection appeared, which gradually lengthened, until the organism somewhat resembled a retort, with the nucleus and pigment occupying the darkly staining, bulbous portion, and a long palely staining, finger-like projection corresponding to the delivery tube (Plate 1: g and t). The nucleus and pigment then gradually moved forward, filling out the finger-like projection.

In the other method of development, which was only observed in *P. vivax*, a projection appeared as before, but, instead of it growing out as an elongated, purely cytoplasmic structure, the nucleus and pigment of the zygote moved forward *pari passu* with its development, and the striking retort-shaped stage was not seen (Plate 1: h).

In warm weather some fully-developed vermicules could be found 14–16 hours after feeding, and by the twenty-fourth hour the majority of the zygotes had reached this stage. Differences in developmental rate were, however, quite obvious even at this period, since it was usual to find some of the earlier stages present at 24 hours, i.e. rounded and retort-shaped forms as well as vermicules. The fastest development was seen in a batch of mosquitoes, which had fed on a *P. falciparum* donor, and which were kept in an incubator at 86° F. (30° C.); a few vermicules were found in them at 10 hours. The fully-formed vermicule measured 14–18μ in *P. vivax*, and 11–15μ in *P. falciparum*. The width in both species was from 2–3μ. It was slightly curved, and one end—presumably the anterior—was narrower than the other (Plate 1: j and v). The pigment was often concentrated at this end. Two nuclei could be found in some vermicules; they were usually well separated from each other, and their significance is not known.

Zygotes, which arose from the unpigmented, or lightly pigmented, *P. falciparum* gametocytes frequently found after atebrin therapy (Plate 1: k), developed into vermicules which differed from those described only in the deficiency of pigment.

Penetration of the gut-wall usually began about the twenty-sixth hour, and was complete by the forty-eighth hour after feeding. Vermicules were occasionally found with one end embedded in a clear, structureless material, which was demonstrable in sections as a definite layer immediately internal to the cubical epithelium lining the gut. Their appearance suggested that they had been actively forcing their way through this layer. Although we have no direct evidence on the point, we consider that penetration is an active process, and not merely a displacement of the cells by growth as Huff (1934) has suggested for *P. relictum* in *Culex pipiens*. We have not detected vermicules in or between the cubical cells in sections, but minute cysts already completely external to the epithelial and muscle layers were found quite regularly at 48 hours, and their measurements did not indicate that much—if any—growth had occurred since the zygote stage.

Growth and Sporulation of Cyst.

Three definite stages could be recognized in the development of the cysts on the wall of the mid-gut.

Stage of growth. The cyst increased in size from about 6 to 50μ in diameter, the contents appeared clear in the living state, except for pigment granules and some relatively large, oily-looking globules. These clear cysts were usually referred to as small, medium or large pigmented cysts according to size. In *P. vivax*, the pigment was usually arranged in a series of fine lines, or scattered irregularly through the cyst. The individual particles were usually small, but occasionally coarse enough to suggest a *falciparum* infection. In the latter species, the pigment was normally in coarse granules, which were frequently arranged in a conspicuous equatorial band. However, when the infection was derived from patients after atebrin therapy all degrees of pigment deficiency were observed, down to a complete lack of it.

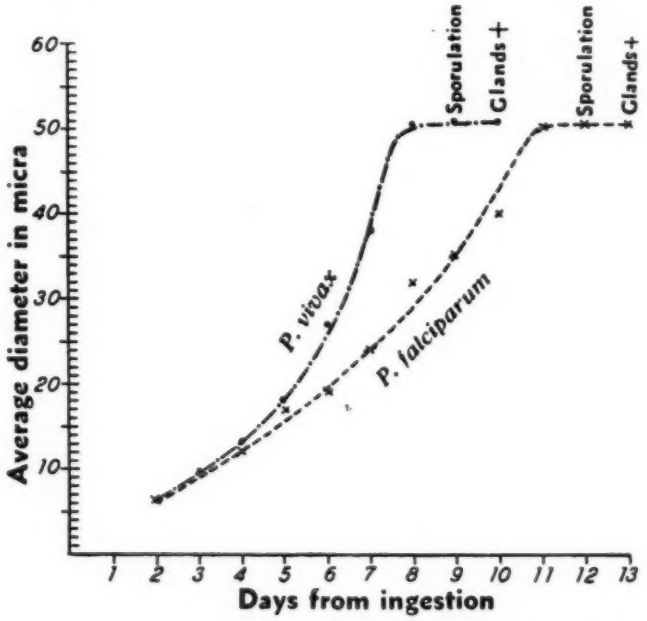


Fig. 1. Growth curves *P. vivax* and *P. falciparum* in *Anopheles punctulatus punctulatus.*

Pre-sporulating stage. The contents appeared opaque in the living cyst, and pigment granules could no longer be detected.

Sporulating stage. The sporozoites were differentiated, first radially from the central mass, and later throughout the cyst.

Growth curves of *P. vivax* and *P. falciparum* cysts developing under similar conditions are presented in Fig. 1. The growth rate of the two parasites was similar until about the fourth day, after which *P. vivax* forged ahead, and reached full size on the eighth day, while sporozoites entered the glands on the tenth day. With *P. falciparum*, growth proceeded more slowly, the cysts reached full size on the eleventh day, and glands were infected on the thirteenth day. The longer developmental period in *P. falciparum* seems to be due to its

relatively slow growth between the fourth and tenth days. Temperatures recorded during the period of observation were:

Mean maximum 88° F. (range of maximum readings 86° to 90° F.).
Mean minimum 73° F. (range of minimum readings 70° to 75° F.).
Mean mean 80·5° F. (27° C.).

We had no real control over temperature during the earlier part of the investigation, when the work was carried out in an improvised laboratory. Humidity was always kept at a high level by wrapping the mosquito cages in wet towels, but it was only later, when better facilities were available, that a more equable climate was ensured by warming the storage room with a radiator in winter, and cooling it in summer by using a fan to increase the evaporation of water from curtains hung around the cages.

The results of two years observations on the seasonal variation in the rate of development of the parasites are set out in Fig. 2. The mean monthly temperatures are shown in relation to the mean developmental period of *P. vivax* and *P. falciparum* from ingestion of gametocytes to the appearance of sporozoites in the salivary glands.

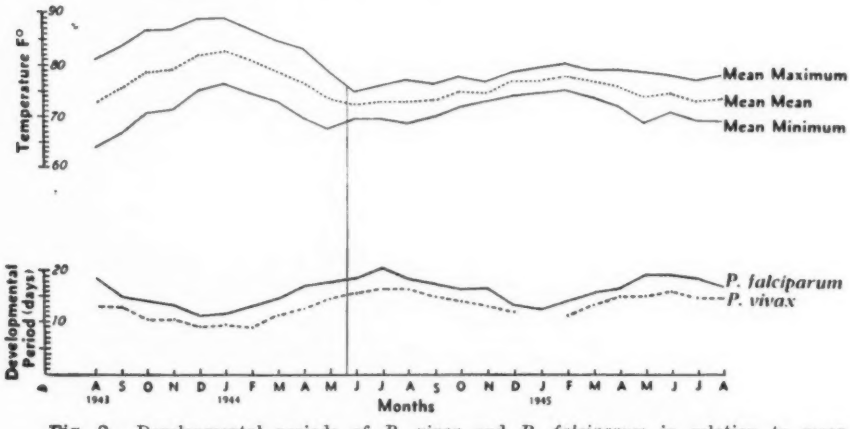


Fig. 2. Developmental periods of *P. vivax* and *P. falciparum* in relation to mean monthly temperatures. The vertical line indicates transference from uncontrolled to partly controlled temperatures.

The most rapid development occurred in the first summer which was unusually hot and dry. With a mean monthly temperature of 82° F. (27·7° C.), the average developmental period for *P. vivax* was slightly over nine days, the minimum observed being eight days. Under the same conditions, *P. falciparum* needed on an average eleven days for complete development, the minimum, observed on one occasion only, being nine days. As the weather became cooler the developmental periods lengthened, and, in mid-winter, *P. vivax* required on an average sixteen days, and *P. falciparum* nineteen days, the mean monthly temperatures being 72·5° F. (22·5° C.). It should be realized that results obtained at these mean temperatures are not comparable with times recorded under constant temperature conditions. Under our conditions considerable fluctuations in temperature occurred, and there were varying periods of enhanced growth and retarded growth which cannot be assessed properly. It may be noted, however, that *P. vivax* developed more quickly than *P. falciparum* at all temperatures studied.

Although the majority of cysts grew at about the same rate, there were always a few which lagged behind, and after about a week it was nearly always possible to find cysts of very different sizes. It is considered that it would be quite impossible in the field to say from an inspection of the gut whether a mosquito had ingested more than one infective blood meal. This subject is discussed more fully elsewhere (Mackerras and Roberts, 1947).

It is often stated that gut infections in *P. vivax* are not as heavy as in *P. falciparum*. This may be true on the average, but we saw many infections with *P. vivax* in which the whole gut-wall was covered with tightly packed cysts, the infestation being as heavy as any seen in *P. falciparum*.

Cysts, which reached full development and burst, left very little trace of their presence. The thin cyst wall collapsed, and empty cysts could be seen for a few hours, but soon all trace of infection disappeared. However, many cysts did not complete their full development, and they left definite scars on the gut, which persisted throughout the life of the mosquito. Death and degeneration of cysts of all sizes were observed. In the earliest stage all that was left was a small brown spot, sometimes rounded and sometimes irregular in outline. Larger cysts retained their shape, but their contents became brown or black. This was thought to be due to the reaction of the insect tissue to a dead foreign body by throwing out chitin in an attempt to wall off the irritating material. Sometimes the result was a solid-looking, black cyst, and at other times the contents became transformed into the so-called Ross's spores. Dead or dying cysts were found at all times of the year, and in practically every batch of mosquitoes, if looked for carefully at the right stage. Some infected batches of mosquitoes showed a high proportion of them.

Sporozoites.

When fully developed, the cyst had a thin, clear, outer wall, through which the densely-packed sporozoites could be seen, often in very active motion. When these cysts burst, the escaping sporozoites displayed their greatest activity. They vibrated rapidly from side to side, but without progressing in a linear direction. It is not known how they reach the salivary glands, but it seems probable that they are carried more or less passively in the haemolymph. When they come in contact with the glands, their energetic vibratory motion may be the means by which they enter the secreting cells. A passive circulation would account for the fact that sporozoites are found in practically every part of the body—legs, palps, etc. All secreting parts of the salivary glands may become infected, but the greatest concentration was usually found towards the base of the glands where the lobes coalesce. Heavily infected glands became swollen and friable, and the normal firm, shining, smooth appearance was lost.

The sporozoites of *P. vivax* and *P. falciparum* are very slender, shining, slightly curved rods about 11–12μ in length. They are similar to one another, but can be distinguished by careful measurement from those of *P. malariae*, which are slightly longer (about 14μ) and thicker. When the crushed salivary glands were examined in physiological saline or in blood, the sporozoites exhibited slow bending and rolling movements, but were never observed to make any forward progress.

Shute (1945) devised a method of counting sporozoites. He demonstrated a decrease in numbers in mosquitoes allowed to feed repeatedly on a rabbit, and considered that a mosquito might inject 3,000 sporozoites at each biting. We have observed a rapid decrease in the heaviness of gland infections in batches of mosquitoes, which had only an occasional blood meal, and in others which had fed on apple juice only.

In one experiment, an attempt was made to calculate the number of sporozoites present, and to obtain some idea, if possible, of the number injected during biting. At that time we were not aware of Shute's method, and the estimation was done by crushing the glands

thoroughly in a small, measured amount of saline, and then running a few drops of the well-agitated suspension into a counting chamber. Counts were made in this way on seven mosquitoes infected with *P. vivax* four days after the salivary glands had been invaded. They ranged from 900–108,000, the average being 46,000 sporozoites per mosquito. Two weeks later, counts were made on six mosquitoes of the same series, which, in the intervening period, had taken three blood meals, as well as having access to fresh apple daily. The range was 9,000–89,000 and the average 32,000 sporozoites per mosquito. However, a control series which had not been allowed to have any blood meals, gave an average of 15,000 (range 120–23,000). The wide range of the readings, and the rapid loss by the controls made it impossible to draw any conclusions about numbers of sporozoites introduced at biting. However, it confirmed what had been observed qualitatively on many occasions, namely, that there is a steady depletion of sporozoites when mosquitoes have access to carbohydrate food only. It seems probable, therefore, that they escape into whatever substance the mosquito feeds upon, although possibly some may die and become absorbed within the glands. In a recent book, Russell, West and Manwell (1946) described a method of detecting infection in a live mosquito by allowing it to suck glucose solution from a cover slip. Sporozoites escape into the fluid and may be seen under the microscope.

The depletion of sporozoites may go on until some mosquitoes become free from infection. This probably only occurs if the original infection was a light one. In one batch of lightly infected mosquitoes the sporozoite rate fell from 40 p.c. shortly after the invasion of the glands to 24 p.c. three weeks later. James (1926) recorded a similar condition in a batch of mosquitoes, in which the sporozoite rate fell from 85 p.c. on the fiftieth day to 20 p.c. on the seventieth day.

It was unusual to find any abnormal sporozoites in a living mosquito, and they usually retained their shape and refractile appearance even though it could be demonstrated that they had lost their power to infect. In two experiments with *P. vivax*, sporozoites eighteen and twenty-eight days old respectively failed to infect susceptible volunteers; all the mosquitoes used were dissected after engorgement, and found to contain apparently normal sporozoites. Abnormalities were, however, occasionally seen, the most frequent being a twisting and coiling of the sporozoite and loss of refractility. At times Ross's spores were found in the salivary glands among healthy looking sporozoites, and may have been chitinized, dead sporozoites.

It would appear that our mosquitoes kept at room temperature, which very rarely fell below 70° F. (21° C.), lost infectivity far more rapidly than other species kept at low temperatures. James (1926) recorded that some mosquitoes kept at 4° to 5° C., remained infective from 29–92 days after the sporozoites had reached the salivary glands. However, Boyd and Stratman-Thomas (1934) recorded a steady decline in infectivity with increasing age of sporozoites. Their figures show that, with sporozoites aged 11–20 days, they had 93 p.c. successful inoculations, but with increasing age the percentage of successes fell progressively, until only 21 p.c. was recorded with sporozoites 41–50 days old, and none at all with those aged more than 50 days. After early failures with related species, we did not attempt to store *A. punctulatus punctulatus* at low temperatures, so have no experience of the effects of cold on the sporozoites of our strains.

SUMMARY.

The methods employed in studying them, and the morphology of the early stages of *P. vivax* and *P. falciparum* in the mosquito are described.

In both species exflagellation was observed in 5 minutes and fertilization in 10 minutes after ingestion of gametocytes. The majority of zygotes had reached the vermicule stage in 24 hours and had penetrated the wall of the mid-gut in 48 hours.

The developmental period was shorter for *P. vivax* than for *P. falciparum* at all seasons. The mean period varied from 9 days (mid-summer) to 16 days (mid-winter) for *P. vivax* and from 11 to 19 days for *P. falciparum* for the same seasons. The minimum periods observed (mid-summer) were 8 days for *P. vivax* and 9 days for *P. falciparum*.

A steady depletion of sporozoites was noted in mosquitoes having access only to carbohydrate food, as well as in those having blood meals in addition.

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OBSERVATIONS ON THE DEVELOPMENT OF HUMAN MALARIAL PARASITES IN THE MOSQUITO

II. FACTORS INFLUENCING INFECTION

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In a previous part we described the changes in form which *Plasmodium vivax* and *P. falciparum* undergo in the mosquito, and the seasonal variation in the rate of development. In this paper we have attempted to assess the factors which influence the establishment of an infection in the insect host.

GAMETOCYTE COUNTS AND AMOUNT OF BLOOD INGESTED.

Various workers have attempted to ascertain the smallest number of gametocytes which would infect mosquitoes. Boyd and Kitchen (1937) were sometimes able to infect mosquitoes with densities of less than ten *P. vivax* gametocytes per c.mm., and Boyd (1942) reported infection of mosquitoes by a good *P. vivax* infector at a time when his gametocytes were sub-microscopic. Green (1929) in Malaya gave a minimum figure of 10 per c.mm. for *P. vivax*, and 42 for *P. falciparum*.

It is obvious that, to become infected, a mosquito must take up at least one mature male and one mature female gametocyte; it is, therefore, interesting to find out how much blood a mosquito can ingest. This has been found to vary considerably with the species, large anophelines naturally taking more than small species. Boyd, Carr and Rozeboom (1938) found that *A. quadrimaculatus* could ingest 3·2 mg. of blood and *A. albimanus* 1·3 mg. James (1931) found that *A. maculipennis* took 1·8 mg. of blood. We found that *A. punctulatus punctulatus* could ingest 1·4 mg. or approximately 1·3 c.mm. of blood.

However, all mosquitoes do not engorge fully, and this happens frequently when the gametocyte carrier is restless. Some partially engorged mosquitoes were found to have taken only 0·7 c.mm., and others with visible blood in the gut had taken as little as 0·4 c.mm.

Mosquitoes taking between 1 and 1·5 c.mm. of blood might possibly become infected, if the gametocyte count was of the order of 2 per c.mm. The lowest *P. vivax* gametocyte count, from which we obtained some infected mosquitoes, was 24 per c.mm., the infection rate being 20 p.c. and the cysts ranging from 1–3 per gut. With *P. falciparum* the lowest count infecting mosquitoes was 30 per c.mm., the infection rate being 58 p.c. and the cysts ranging from 2–10 per gut. Occasionally quite good infections were obtained with this species (*P. falciparum*) with counts less than 100 per c.mm. For example a batch of mosquitoes with a 94 p.c. infection rate was obtained from a donor with only 40 gametocytes per c.mm.; the average number of cysts, however, was only 5 per gut. These cases were, however, very rare, and we preferred to use donors with at least 200 gametocytes per c.mm.

In Tables 1 and 2 consecutive batches of mosquitoes are grouped according to the gametocyte counts of the donors, the percentage of each group falling in three grades of

infection (nil, 1–50 p.c., 51–100 p.c. infected) being set out. It will be seen that a rise in the gametocyte count was associated in a general way with a rise in infectivity, but there was no absolute correlation between numbers and infectivity, patients with high counts sometimes being non-infective, or only slightly infective.

TABLE 1.

P. vivax. *Relation between gametocyte counts and gut infections in 182 consecutive batches of mosquitoes.*

Gametocyte counts per c.mm.	Number of batches	Gut infection rates		
		Nil.	1–50 p.c.	51–100 p.c.
		Percentage in each grade		
1–100	12	50	33	17
101–200	32	22	18	60
201–400	47	6	19	75
401–1,000	75	8	14	78
1,001–2,000	16	—	19	81

TABLE 2.

P. falciparum. *Relation between gametocyte counts and gut infections in 446 consecutive batches of mosquitoes.*

Gametocyte counts per c.mm.	Number of batches	Gut infection rates		
		Nil.	1–50 p.c.	51–100 p.c.
		Percentage of batches in each grade		
1–100	20	25	20	55
101–200	60	8	22	70
201–400	72	12	10	78
401–1,000	131	5	7	88
1,001–2,000	87	3	6	91
2,001–4,000	43	9	5	86
4,001–8,000	24	0	4	96
>8,000	9	0	0	100

The higher counts were usually associated with a heavier infestation of the gut, but even this was not a constant finding, confirming the work of James, Boyd, and other observers that the quality of the gametocytes is more important than their number.

THE QUALITY OF THE GAMETOCYTES.

P. VIVAX.

In *P. vivax* infections, gametocytes usually appeared in small numbers about six days after the first appearance of trophozoites, but, as a rule, it was not until the third or fourth day of the gametocyte wave that sufficient were present to warrant feeding mosquitoes. If abundant by the third day, they were commonly infectious to mosquitoes.

Among our patients were men who infected mosquitoes whenever tested, and others who were highly infective on one day and practically non-infective on the next day, although the gametocyte count remained high. These changes in infectivity could not be correlated with morphology or sex ratio; nor could they be associated with a particular type of fever; nor with differences in the mosquitoes, which all came from the same stock.

Three examples of sudden changes in infectivity are set out in Table 3. Four batches were for all practical purposes not infected (i.e. from 18–27 consecutive negative dissections were recorded). Three of the non-infected batches were obtained on the day therapy with quinine or atebrin began, but in two cases the mosquitoes were applied before the first dose of drug was given, and we had evidence that neither drug acts on mature gametocytes.

TABLE 3.

P. vivax. *Variations in infectivity in same carriers.*

Patient	Day of gameto-cyte wave	Time	Game-to-cytes per c.mm.	Per-cent-age of males	Gut infection		Gland infection		No. of nega-tive dissec-tions
					Per-cent-age in-fected	Aver-age cysts per gut	Per-cent-age in-fected	Type of infection	
A.P.	4	1900 hrs.	960	50	—	—	—	—	27
	5	1900 hrs.	500	47	83	5	83	medium	
	6*	1100 hrs.	800	49	—	—	—	—	20
	7	1000 hrs.	650	40	100	43	100	heavy	
		1830 hrs.	650		79	8	70	light-medium	
	8	1000 hrs.	400	50	60	10	44	light	
W.C.C.	2	1900 hrs.	310	53	58	7	58	light	
	3	1500 hrs.	390	64	100	45	100	heavy	
	4*	1030 hrs.	360	55	—	—	—	—	18
	5	1900 hrs.	620	45	90	20	50	light	
	6	1830 hrs.	510	47	4	1	—	—	
G.W.H.	5	1900 hrs.	680	—	100	50	92	heavy	
	6*	1130 hrs.	720	41	—	—	—	—	27
		1900 hrs.	920	43	100	27	82	medium-heavy	
	7	1130 hrs.	850	29	93	50	93	heavy	
		1930 hrs	680	26	100	17	100	heavy	
	8	1130 hrs.	550	23	100	7	82	light	
		2000 hrs.	200	26	82	7	81	light	

* Treatment begun.

Gut smears had been made at 20-minute and 24-hour intervals from these batches. In the early smears from the non-infected batches, extracellular female gametocytes were quite abundant, and many were normal in appearance. No exflagellated male cells were seen, however. In late smears (24 hours) no vermicules were found in the batches from A.P. and W.C.C., but in a mosquito fed on G.W.H. a few were observed. These findings were in marked contrast to those recorded for the well-infected batches, in which numerous exflagellating males were seen, and in the late smears considerable numbers of vermicules. It seems obvious that the failure of the gametocytes to infect the mosquitoes was due to some inherent fault within themselves, and not to any factor operating in the insect host. It is possible that there were successive waves of gametocytes, each of which circulated for a short period only, and had even shorter periods of maturity.

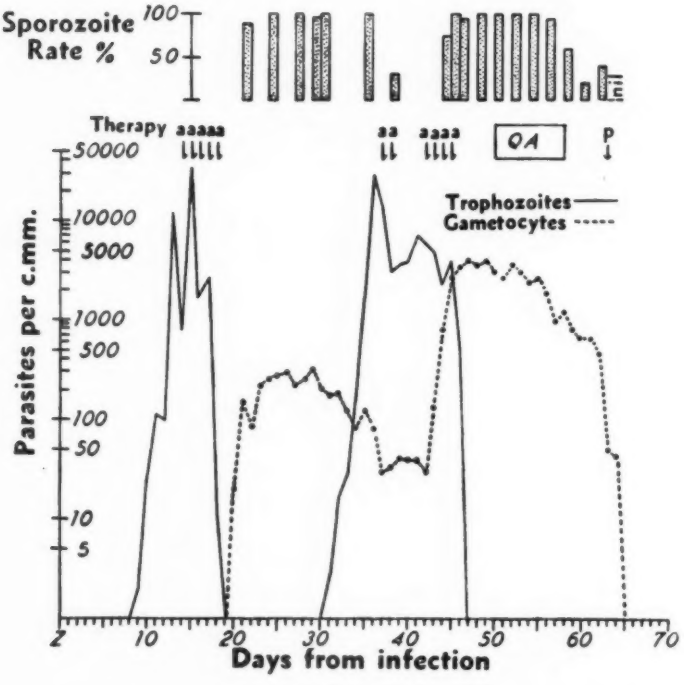

Fig. 1. Trophozoite and gametocyte waves in patient C.D.S. bitten by 13 infected mosquitoes.

Therapy. a = atebrin 0·5 gm. on days 14 and 16; 0·3 gm. on day 15; 0·1 gm. on days 17 and 18; 0·3 gm. on day 37; 0·1 gm. on day 38; 0·05 gm. on days 42 and 43; 0·3 gm. and 0·4 gm. on days 44 and 45; Q.A.* began on day 50; p = plasmoquine 10 mg. on day 62.

* Q.A. = the first 8 days of the standard course of treatment, namely quinine 2 gm. daily for 3 days, atebrin in decreasing doses from 0·6 gm. to 0·2 gm. for 5 days. The full course, Q.A.P., concludes with quinine 1 gm. and plasmoquine 30 mg. daily for a further 5 days.

P. FALCIPARUM.

Maturity in Relation to the Gametocyte Wave.

In *P. falciparum* infections, gametocytes usually appeared about 10 days after the first trophozoites were detected. At the beginning of the wave spindle-shaped gametocytes were often observed, and we presumed that they were non-infective, as we never saw them round up or exflagellate, and mosquitoes fed during the first two or three days were often very poorly infected, or not infected at all. However, infection on the first day of the gametocyte wave could sometimes be obtained. For example, one patient had a count of 600 gametocytes per c.mm. on the first day and all the mosquitoes fed on him became infected; these gametocytes were of the usual crescentic shape, but many were apparently immature as large numbers of the cysts which formed died before reaching maturity, and the sporozoite rate of the batch was only 42 p.c.

From the fourth day of the wave until about the tenth day, very heavy infections were usually recorded, and, in our experience, patients were usually either very good or very bad infectors at this stage. From the tenth day onward, infectivity usually declined in an irregular way, and towards the end of the wave most carriers became non-infectious. There were some notable exceptions, in whom gametocytes remained infectious practically throughout the wave (Fig. 1).

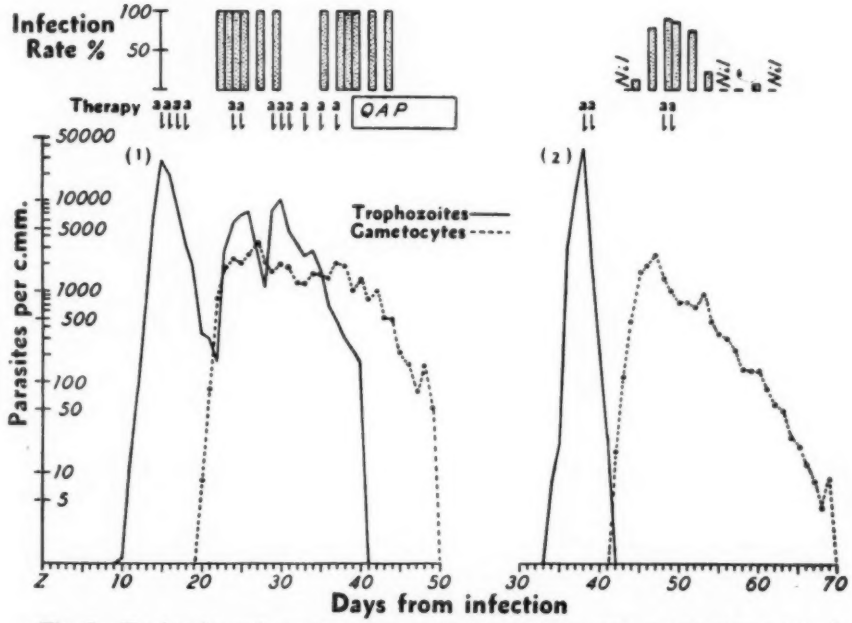

Fig. 2. Trophozoite and gametocyte waves in two patients, each experiencing a second clinical attack with the same strain. (1) Prolonged trophozoite wave; (2) short trophozoite wave.

Therapy. (1) a = atebrin 0·1 gm. on days 15 and 16; 0·05 gm. on days 17, 18, 24, 25, 29, 30, 31, 33, 35, and 37; Q.A.P. commenced on day 39. (2) a = atebrin 0·5 gm. on days 38, 39, 48, and 49.

A distinction must be drawn between patients whose trophozoites were eliminated, and those in whom they were allowed to continue at moderate densities, the latter being controlled by small doses of atebrin. In the former group, gametocytes reached a peak, and then steadily declined, usually both in numbers and infectivity, whereas in the latter each upward fluctuation of trophozoites was followed about ten days later by an increase in gametocytes. The gametocyte wave then resembled a plateau, and these patients remained highly infectious for long periods. Comparison of the two types may be seen in Fig. 2.

The longest periods at which some infective gametocytes were found to persist without obvious replenishment were: (1) the twenty-seventh day of the gametocyte wave and the twentieth after the disappearance of trophozoites, gametocytes being 90 per c.mm. and the sporozoite rate 28 p.c.; and (2) the twenty-fifth day of the gametocyte wave and the twenty-seventh after the disappearance of trophozoites, gametocyte 120 per c.mm. and sporozoite rate 25 p.c.

Variation in infectivity.

Variation in infectivity from day to day, which was frequently observed in *P. vivax*, was not a feature of *P. falciparum* infections. In one patient, however, we observed a sudden loss of infectivity just as the count was beginning to decrease. The details are set out in Table 4.

TABLE 4.

P. falciparum. *Infection rates in mosquitoes and corresponding gametocyte counts in patient showing a marked change in infectivity.*

Day of gametocyte wave	Gametocytes per c.mm. (approx.)	Gut infection rate (p.c.)
4	650	100
5	500	7
6	250	Nil
9	200	Nil

Consistently Non-Infectious Gametocytes.

The most puzzling feature encountered in our work with *P. falciparum* was the occurrence of gametocytes which consistently failed to infect mosquitoes. One patient (W.E.T.), who turned out to be a non-infector, was admitted early in the gametocyte wave, and we fed mosquitoes during the peak, i.e. the period normally found to be most infectious. Careful examination of stained blood films failed to reveal any abnormality in his gametocytes, which might be correlated with failure to infect mosquitoes. When smears from the mid-gut of mosquitoes were examined, however, and compared with a parallel series from a good infector, a great difference in the behaviour of the gametocytes was observed.

Many gametocytes rounded up, became extracellular, and at first appeared quite normal in the living state. However, very few exflagellating males were seen, and in the stained films of mid-gut contents made at frequent intervals after feeding, *free* microgametes were never detected. Those that were seen were still attached to the parent cell, they appeared thin, and stained palely and irregularly. Macrogametes were hard to find in mid-gut smears, except on one occasion when they were found in four mosquitoes dissected, varying in number from 50–130 per gut. It was noted that none of them contained two nuclei. In films made 30–45 minutes after feeding many showed obvious signs of disintegration, the outline

being irregular, and the cytoplasm vacuolated. Presumably in other mosquitoes they had disintegrated even earlier. No vermicules were detected in any batch. Phagocytosis was recorded as very active on several occasions, and undoubtedly accounted for many gametocytes. The most remarkable feature was the great activity of the eosinophils, which were fairly abundant in this patient, sometimes forming 15 p.c. of the total white cells. In one gut smear made thirty minutes after feeding approximately the same proportion of eosinophils and neutrophils contained parasites, 15 p.c. of 125 eosinophils, and 16 p.c. of 240 neutrophils. However, similar activity of the neutrophil leucocytes was frequently noted in good infectors with a lower total gametocyte count, and yet sufficient zygotes survived to produce a heavy infection.

The failure of W.E.T.'s gametocytes to infect mosquitoes seemed to be due to some inherent functional peculiarity, the cause of which is quite obscure. Details of the behaviour of these gametocytes, as far as is available, are set out in Table 5.

TABLE 5.

Behaviour of gametocytes in a non-infector W.E.T.

Days from admission	Gametocytes per c.mm.	Males (p.c.)	Exflagellation	Extracellular female gametocytes per gut	Phagocytosed gametocytes per gut	Vermicule formation	Number of negative gut dissections
2	140	n.d.	nil	nil	n.d.	—	17
8	2,300	32	very scanty	nil	800	—	22
9	2,300	48	very scanty	scanty	900	—	33
11	1,300	n.d.	n.d.	n.d.	n.d.	n.d.	10
12	2,500	50	very scanty	50–130	300	—	32
13	1,700	64	n.d.	n.d.	n.d.	n.d.	12
21	1,000	n.d.	abortive	scanty	present	—	10
Total dissections							136

n.d. = not determined.

A similar set of events was recorded in a volunteer experiencing his third clinical attack, and for whom full details of date of infection, trophozoite counts and therapy were available. Lack of infectivity could not be correlated with age of gametocytes, nor with therapy. Mosquitoes were fed on six occasions, and in one batch a 6 p.c. sporozoite rate was recorded, but no other infected mosquitoes were found in 168 dissections. Gametocyte counts ranged from 400–940 per c.mm. In gut smears, exflagellation was very seldom observed, macrogametocytes rapidly degenerated, and vermicules were never seen. This patient had infected mosquitoes quite freely during his second attack a month previously, the findings on one day being: gametocytes 560 per c.mm., sporozoite rate 83 p.c. There seemed no reason at all why the next wave of gametocytes should be almost entirely non-functional.

FACTORS REDUCING INFECTION IN THE MOSQUITO.

When counts of apparently viable organisms were made in gut smears at intervals during the first 24 hours after feeding, and compared with one another and with the number of cysts subsequently found, a fairly steady loss in numbers was usually observed. For example, in one *P. vivax* infection the patient's gametocytes were estimated at 650 per c.mm., 60 p.c.

or 390 being females. A mosquito imbibing 1·3 c.mm. of blood, would then have ingested about 520 female gametocytes. In a gut smear made at 20 minutes after feeding, extracellular female gametocytes numbered 360 per gut; in a 24-hour smear there were 220 vermicules; and in mosquitoes dissected subsequently the number of cysts varied from 4 to about 150, the average for 11 dissections being about 40 per gut, i.e. less than 10 p.c. of the original female gametocyte count.

Phagocytosis.

The phagocytic activity of the human white cells was an important factor in reducing the number of viable organisms. It was observed for several hours after the blood had been ingested by the mosquito. It was also studied in fresh blood preparations sealed with vaseline and kept at room temperature. Although both eosinophils and monocytes were observed with the remains of parasites in them, the greatest activity was found in the neutrophils, which were repeatedly observed to pass over gametocytes contained in red cells and to absorb only those which were extracellular.

On one occasion, a neutrophil was observed to engulf two within an hour, and on another occasion two within four hours. Activity was observed for five hours *in vitro* at room temperature. Living gametocytes of both sexes were attacked, including exflagellating males. A neutrophil, which was observed in the act of engulfing an exflagellating male gamete, then proceeded to move among the red cells with a bunch of still active "flagella" projecting from it. At first the ingested gametocyte appeared normal and the pigment remained in motion, after about 35 minutes the pigment ceased to dance, and in one hour signs of digestion of the gametocyte appeared. After five hours the gametocyte had disappeared, and only scattered pigment granules were left.

Since high gametocyte counts were sometimes recorded with *P. falciparum*, there was often more scope for phagocytosis of this species, and it was a very marked feature of many gut smears. Sometimes a loss of 10 p.c. of the gametes had occurred in the first 30 minutes. It was thought possible that variations in infectivity could be correlated with fluctuations in leucocytes, and one attempt was made (with inconclusive results) to test this by increasing the leucocyte count artificially.

In this experiment mosquitoes were fed immediately before and ten minutes after the patient received a hypodermic injection of 1 ml. adrenalin (1 in 1,000). Leucocyte and gametocyte counts were made at the same intervals. The gametocyte count was unchanged, 1,400 per c.mm., but the leucocytes rose from 7,600 to 17,600 per c.mm. In spite of this increase in numbers no increase in phagocytosis could be demonstrated; the actual figures were, indeed, slightly lower after adrenalin. Both batches of mosquitoes became heavily infected and to approximately the same degree, the number of cysts per gut varying from 17 to about 100.

Darling (1910) was one of the first observers to point out the importance of phagocytosis in limiting the infection in the mosquito. During the first few hours in the gut of the mosquito it is a major hazard in the life of the parasite. It helps to account for minor variations in the infective rates of mosquitoes, but not major ones like those seen in Table 3, where extraordinary fluctuations in leucocytes would be necessary to account for the results.

Other factors reduce the numbers of parasites later in development. In one batch of mosquitoes fed on a *P. vivax* gametocyte carrier, the occurrence of numerous, minute, brown spots on the gut wall was a striking feature, which suggested that vermicules had penetrated the wall successfully, but had failed to become encysted. This batch became only lightly infected. In many batches it was common to observe that death and degeneration of a proportion of the organisms, and formation of Ross's spores, had occurred. It was a marked

feature with *P. falciparum* very early or late in the gametocyte wave. In these batches the sporozoite rate was much lower than the gut infection rate.

It appears, then, that there are intrinsic and extrinsic factors influencing the establishment of infection in the mosquito. Among the former is the variable viability of the parasite itself, and among the latter the phagocytic activity of the human white cells and a variable resistance on the part of the mosquito. To these must be added the mechanical hazard associated with the necessity of the microgamete finding a macrogamete within a limited time. This hazard becomes greater when the total count is low, and the macrogametes are scattered widely through a relatively large mass of blood. With regard to the resistance of the insect, we have shown elsewhere that even in a highly susceptible species like *A. punctulatus punctulatus* variations in susceptibility do occur (Mackerras and Roberts, 1947).

EFFECT OF THERAPY.

This has been fully dealt with elsewhere, and we need only recapitulate the findings here. Neither quinine nor atebrin produced any morphological change in mature *P. vivax* or *P. falciparum* gametocytes, and they continued to infect mosquitoes while therapeutic doses were being taken. The action of paludrine was essentially different from that of quinine or atebrin. It did not prevent growth of young forms and caused no morphological change in mature gametocytes. However, young cysts invariably died, so that complete sterilization of the infection in the mosquito occurred (Mackerras and Ercole, 1947). Plasmoquine had a marked action upon *falciparum* gametocytes, sterilizing them in 15 hours, and removing them from the circulation in about 3 days (see also Figs. 1 and 2). *P. vivax* gametocytes appeared less sensitive to the action of plasmoquine than those of *P. falciparum*.

SUMMARY AND CONCLUSIONS.

Infection in the mosquito depended chiefly on the maturity of the gametocytes. This quality could be best assessed by observing their behaviour in the gut of the mosquito 15 to 30 minutes after ingestion. If vigorous, free microgametes were found, subsequent infection in the mosquito was always observed. When the male cells failed to exflagellate, the females frequently appeared normal at first, and may have been functional. Unfertilized macrogametes, however, quickly disintegrated and disappeared.

Numbers *per se* were no guarantee of infectivity, but in general the higher the gametocyte count the better the infection in the mosquito in both species.

Failure to infect mosquitoes could not be correlated with any factor in *P. vivax*. Marked changes in infectivity in this species were recorded in as short a period as 8 hours, and were commonly observed to occur within 24 hours. These changes were thought to be due to the formation of successive waves of gametocytes, which circulated for a short period only, and had an even briefer span of infectivity. No *consistent* non-infectors were observed among *P. vivax* carriers infected with New Guinea strains.

In *P. falciparum* there was usually a period of low infectivity at the beginning of the gametocyte wave, and, in some patients at least, this was correlated with the occurrence of young, spindle-shaped gametocytes. Infectivity

was usually greatest at the peak of the wave, and then declined gradually, and sometimes disappeared while the count was still high. Other patients were highly infectious throughout the wave.

One *P. falciparum* carrier consistently failed to infect mosquitoes although the total counts were adequate, and another produced an extremely light infection on one occasion only. In these men the male gametocytes seemed to be almost entirely non-functional. This lack of function could not be correlated with any other factor.

The parasite suffered a progressive loss in numbers after ingestion by the mosquito. When total counts were low, the mechanical difficulties of the microgamete in finding a macrogamete were increased, and many of the latter failed to become fertilized. Leucocytes of the human host continued to phagocytose extracellular parasites for about 5 hours *in vitro*; they were active also after ingestion by the mosquito, and were responsible for the destruction of many gametocytes and zygotes. Neutrophils displayed the greatest activity, but monocytes and eosinophils also ingested gametocytes.

A varying proportion of vermicules failed to penetrate the gut wall, and, of those which successfully formed cysts, some usually died and became chitinized before sporozoites were formed.

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THE GLYCOGEN CONTENT OF RABBIT HAIR

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Mammalian hair, in addition to the insoluble protein keratin, contains a water extractable fraction (Bolliger and McDonald, 1947a). Uric acid, one of the recognized constituents of this fraction, has been previously reported upon (Bolliger and Hardy, 1945). Another constituent, glycogen, recently has been identified in the fur of the rabbit and a similar substance, though usually in smaller amount, has also been observed in the keratinous appendages of the integument of a number of mammals, including man. In this communication particular emphasis will be placed on the glycogen present in the fur of the rabbit.

METHODS AND RESULTS.

Extraction of Water Soluble Fractions.

The fur of rabbits, living or recently killed, was clipped off fairly close to the skin and after weighing was extracted with water, with or without a preliminary defatting with alcohol and ether. For large amounts of hair two methods of extraction were employed.

(a) The hairs were placed in a muslin bag of suitable shape and were extracted in a Soxhlet apparatus. About ten hours of continuous extraction with water were found to be adequate. If necessary, the water extract thus obtained was concentrated *in vacuo* to a convenient volume.

(b) When dealing with large amounts of hair for which the Soxhlet apparatus employed was too small, the hair was boiled for about twenty minutes in water sufficient to prevent serious bumping. This extract was decanted and as much liquid as possible removed from the hairs by filtration under pressure. This process was repeated six times. The combined extracts were then concentrated *in vacuo*. This method usually furnished an almost colourless concentrate.

Similar extraction was effected at room temperature by adding water to the hairs and wetting them by means of vigorous shaking and stirring. This was usually repeated at least three times. The combined filtrates were then concentrated *in vacuo*.

Isolation of Glycogen.

Solid sodium hydroxide was added to the concentrated filtered extract until it reached a strength of about 50 p.c. After being heated on a water bath for three hours, the solution was filtered and nine volumes of absolute alcohol added to the filtrate. The solution was then allowed to stand in the refrigerator overnight. The white flocculent precipitate formed which subsequently was shown to consist mainly of glycogen and sodium carbonate was collected on

a filter and washed with alcohol and ether. Purification was effected by redissolving it in water, making faintly acid with acetic acid and then re-precipitating with alcohol. Addition of a large volume of alcohol to the concentrated extract, without caustic soda treatment also brought down a voluminous amorphous precipitate. From different samples of fur these methods yielded 300–800 mg. p.c. dry substance which was later proven to consist to a large extent of glycogen.

Identification of Glycogen.

The white amorphous precipitate obtained from aqueous extracts of rabbit fur was proven to be mainly glycogen by the following tests:

(1) On micro-analysis it was found to consist of carbon, hydrogen and oxygen only.

(2) It was readily soluble in water from which it was precipitated by alcohol. It was not attacked by 60 p.c. sodium hydroxide solution at 100° C. It was soluble in half-saturated ammonium sulphate but was precipitated on full saturation.

(3) On the addition of dilute iodine (Lugol) solution to an aqueous solution of the solid, the red colour characteristic of glycogen was observed. This colour disappeared on heating the solution but returned again on cooling. The addition of alkali destroyed the colour but acid had no effect.

(4) The substance gave a positive Molisch test, but had no reducing properties. However, it reduced Benedict's reagent after hydrolysis with mineral acid or with amylase prepared from rat parotid glands. The latter hydrolysates, after neutralization, gave no colour with dilute iodine solution. The product of acid hydrolysis was identified as glucose by means of its osazone and by the characteristic colours and spectrophotometric curves obtained on heating with thymol and with catechol (Bolliger and McDonald, 1947b).

Determination of the Glycogen Content of Rabbit Fur.

An aliquot of the concentrated filtered water extract was hydrolysed with mineral acid and resulting reducing substances were estimated as glucose by the method of Shaffer, Hartmann and Somogyi (1926). However, reducing substances were also found to be present in the extract prior to this hydrolysis. Accordingly, a determination was carried out before and after heating with acid and the difference between these values times 0·912 was taken as the amount of glycogen in the sample.

Example: 2 gm. of rabbit fur placed in a covered extraction thimble were extracted with water in a Soxhlet of suitable size for a period of eight hours. The filtered extract was then concentrated by heating to a volume of 20 ml. Two 5 ml. aliquots of this concentrate were then transferred to large test tubes and about 0·2 ml. concentrated hydrochloric acid was added to one tube. The tubes were then placed in a boiling water bath for one hour, care being taken not to allow the volumes to fall below 1–2 ml. After neutralizing the acid with solid sodium bicarbonate, 5 ml. of the Shaffer-Hartmann-Somogyi reagent (1926) were added to each and the determination of the reducing substances present was carried out as usual. The glucose content of the unhydrolysed sample was subtracted from that found for the hydrolysed specimen. Results thus obtained are listed in Table 1.

Removal of Glycogen from the Living Animal.

A live Angora rabbit was placed in a large bowl, and a known amount of water at 30–35° C. was poured over it until it was covered except for the head and neck. After leaving the animal immersed for ten minutes and rubbing the hairs to wet them, it was removed and

glycogen and reducing substances were estimated in the water after filtration and concentration. The amount of reducing substances was 11 mg., and the amount of glycogen 28 mg.

This was repeated after one week. The amount of immediately reducing substances was found to be 13 mg., and the amount of glycogen 13 mg. In a short-haired rabbit the glycogen content of the washwater was found to be 4 mg. and the immediately reducing substances 3 mg.

TABLE 1.

Rabbit.	Mode of extraction of hair.	Before hydrolysis. Glucose mg. p.c.	After hydrolysis. Glucose mg. p.c.	Difference glucose mg. p.c.	Glycogen mg. p.c.
1	Cold	35	98	578	528
	Hot	114	629		
2	Cold	59	140	327	298
	Hot	96	342		
3	Soxhlet	148	596	448	409
4	„	178	557	379	345
5	„	332	730	398	362
6	„	216	668	452	412
7	„	181	544	363	331
8	„	225	575	350	319

Amount of reducing substances and glycogen present in rabbit fur. The figures listed were determined on the fur directly as obtained from the animal, without preliminary drying or defatting.

DISCUSSION.

Histological studies, initiated more than forty years ago, showed that the glycogen of the skin is deposited almost exclusively in the lower half of the outer root sheath of the hair follicle (Brunner, 1906; Sasakawa, 1921). This probable relationship between skin glycogen and the keratinous integument has not been followed up and the suggestive histological observations have apparently been forgotten.

Recent investigations on the skin of the rabbit have not only confirmed by means of staining reactions the presence of large amounts of glycogen in the outer hair root sheath, but they have also shown that glycogen is, in addition, deposited in the lower parts of the hair shaft near the papilla (Bolliger and McDonald, 1948). On the other hand, it has not yet been possible to demonstrate histologically the presence of glycogen in the extradermal hair, but the experi-

mental findings recorded in this paper indicate that glycogen is present in this hair in unexpectedly large amounts (300–500 mg. p.c.), as proven by chemical and enzymatic assay.

Furthermore, the water extract of rabbit fur was found to contain substances reducing immediately without previous hydrolysis. It is not yet known if these substances are directly related to the glycogen present in the same location, a breakdown of which has not yet been observed on clipped hair where the glycogen content and the directly reducing substances remained apparently stable for several weeks.

A fraction of the glycogen as well as of the immediately reducing substances is readily removed from the clipped hair by water at room temperature (Table 1). The same also applies to the fur of the living animal, which therefore may lose oxidizable carbohydrates when it is drenched with rain. It will certainly also lose glycogen when it sheds hair, as for example during the moulting season.

So far the significance of the glycogen in hair is not known. On histological evidence it seems to play an important rôle in the formation and maintenance of the deeper sections of the outer root sheath and of the hair root. On keratinization and concurrent dehydration the supply of glycogen to the hair seems not to cease, and in the absence of experimental proof it may be assumed that this reserve carbohydrate, originally present in large liquid droplets according to histological preparations, becomes deposited on the keratin micelles of the hair fibre.

So far Pflueger's method of isolating glycogen from tissues by means of digestion with strong alkali could not be successfully applied to fur directly because it appeared that additional reducing substances formed from the digested keratin led to erroneous results. Digestion of the filtered aqueous hair extracts with strong sodium hydroxide was found to be a more satisfactory method of isolation.

The results given in this paper for the glycogen content of untreated rabbit fur show considerable variations in different animals. However, duplicate experiments on the fur of the same rabbit showed differences up to 20 p.c.

The possible causes for these discrepancies, such as mode of extraction, variations in the glycogen content of hair from different body regions, etc., cannot yet be discussed and so far we have only succeeded in establishing the approximate amount of glycogen present in the fur of the rabbit.

SUMMARY.

Considerable amounts of glycogen (300–500 mg. p.c.) and of directly reducing substances (100–300 mg. p.c.) have been found in the fur of the rabbit. The identity of the glycogen was established by chemical and by enzymatic methods. The glycogen content was determined by measuring the reducing power expressed as glucose of a known amount of aqueous fur extract before and after hydrolysis. The difference between these two values was considered to be glycogen, an assumption supported by the actual isolation of glycogen in an amount of about the same order.

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